

## AN ABSTRACT OF THE THESIS OF

Jianya Huan for the degree of Master of Science in Animal Science presented on March 28, 1991

Title: Effects of Dietary Pyrrolizidine Alkaloids on Copper and Vitamin A Metabolism in the Chicken and Japanese Quail

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Abstract approved: \_\_\_\_\_

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Objectives of this study were to examine effects of dietary pyrrolizidine alkaloids (PA) on copper and vitamin A metabolism in the chicken which are very susceptible to the hepatotoxic effects of PA and Japanese quail which are highly resistant to PA. Also, the possible interaction between copper and vitamin A in the two species and effect of PA on retention of previously stored vitamin A in the chicken were investigated. Three experiments were designed. Experiment 1 was to examine the effect of feeding the PA-containing plant tansy ragwort (TR)(*Senecio jacobaea*) on tissue levels of copper and vitamin A in the chicken. Experiment 2 was to investigate if dietary PA affected the retention of previously stored vitamin A in the chicken. Experiment 3 was to determine if hepatotoxic effects of PA are necessary to influence copper and vitamin A metabolism in Japanese quail.

In experiment 1, a 2x2x2 factorial design with dietary 0 and 5% TR, 0 and 250 ppm copper, and 0 and 25,000 IU/kg diet vitamin A was used. The results showed that body weight gain was reduced ( $P < 0.01$ ) in birds fed TR. Both serum and liver copper

concentrations were markedly increased ( $P < 0.01$ ) in the TR-fed group with 250 ppm copper supplement. Zinc concentrations in the serum and liver were significantly decreased ( $P < 0.05$ ) in the TR-fed groups compared to TR-free groups. Liver iron was increased ( $P < 0.05$ ) in the TR-fed birds. The serum vitamin A levels were significantly decreased ( $P < 0.01$ ) in all TR-fed groups. The ranges of decrease were from 62 to 72 % in four TR-fed groups. The liver vitamin A concentrations were also significantly decreased ( $P < 0.05$ ) in TR-fed groups without vitamin A supplement. The effects of PA on liver and blood vitamin A concentration may reflect PA inhibition of synthesis of retinol-binding proteins, or impaired vitamin A absorption from reduced biliary excretion. There was no interaction between dietary copper and vitamin A levels and tissue concentrations of these nutrients.

In experiment 2, a two period experiment was carried out. In the first period, two groups of chicks were fed a diet containing 25,000 IU vitamin A/kg diet for two weeks followed by a control or TR-containing diet for four weeks. Blood samples were taken at 4 day intervals for 24 days. It was found that by day 8, serum vitamin A levels were significantly depressed ( $P < 0.05$ ). After 24 days of PA exposure, serum vitamin A levels were reduced by 55% and 8.5% in the TR-fed group and the control group, respectively. Liver concentration of vitamin A was increased ( $P < 0.05$ ) at day 24 of TR feeding, while liver vitamin A concentration in birds fed the control diet was decreased by 13% over the same period. The results indicate that PA inhibit the mobilization of previously stored vitamin A from the liver, probably by inhibiting hepatic synthesis of retinol-binding proteins.

Experiment 3 was a 2x2x2 factorial design with added 0 and 5% tansy ragwort, 0

and 250 ppm copper, and 0 and 25,000 IU/kg diet vitamin A. The results showed that consumption of TR did not affect the growth rate of Japanese quail. There were no significant differences in the serum copper concentrations among all treatment groups. Liver copper levels were decreased with TR feeding ( $P < 0.05$ ). The concentrations of zinc and iron in the serum and liver were normal in TR-fed groups compared to the controls. There was no significant effect ( $P > 0.05$ ) on the serum vitamin A concentration. The liver vitamin A concentrations were also not significantly different with the exception of the basal TR group. Copper supplementation of the diet increased serum vitamin A levels ( $P < 0.05$ ). The results suggest that hepatotoxicity is necessary to induce the changes in tissue levels of copper and vitamin A seen in PA-susceptible species.

**Effects of Dietary Pyrrolizidine Alkaloids on Copper and  
Vitamin A Metabolism in the Chicken and Japanese Quail**

by

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Dedicated to my sponsor and friend,

Senator Mae Yih

for without her encouragement and concern  
and financial support throughout my graduate program,  
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# **Effects of dietary pyrrolizidine alkaloid on copper and vitamin A metabolism in the chicken and Japanese quail**

## **Introduction**

Pyrrolizidine alkaloids (PA), a large class of naturally occurring toxicants produced by numerous plant species worldwide, are responsible for many cases of livestock losses and some human poisonings (Cheeke and Shull, 1985; Mattocks, 1986). Most PA-containing plants that have caused livestock poisoning are in the genera *Senecio*, *Crotalaria*, *Heliotropium* and *Echium* (Cheeke and Shull, 1985). PA derived from these plant species cause a number of characteristic toxic effects in animals and are mostly noted for their ability to produce hepatotoxicity. The main symptoms of hepatotoxic effects of PA are swollen hepatocytes, progressive fibrosis, bile duct proliferation, veno-occlusion and loss of hepatic metabolic function (Cheeke and Shull, 1985). The main metabolic routes for PA are dehydrogenation to pyrroles, conversion to N-oxides and hydrolysis (Mattocks, 1986). The first pathway yields the toxic pyrrole derivatives, which through covalent binding and subsequent inactivation of essential biological nucleophiles such as protein or nucleic acids which could result in alterations of cell function and lead to cell damage or cell death (Mattocks, 1986). The N-oxidation and hydrolysis of PA are thought to represent detoxification mechanisms (Mattocks, 1986).

PA also have important interactions with nutrients. PA exposure results in elevated liver copper and iron, and decreased zinc in several species (Bull et al., 1956; Swick et

al., 1982a, 1982b; Garrett et al., 1984; Van der Watt et al, 1972). The postulated mechanisms have been suggested to induce impaired subcellular copper excretory mechanisms, lysosomal defects and PA-induced change in the structures of copper binding proteins (Swick et al., 1982a, 1982b). Recently, effects of PA on plasma and liver vitamin A levels in the rat have also been reported (Moghaddam and Cheeke, 1989). These authors postulated that PA affect hepatic retinol-binding protein synthesis and impair biliary excretion, resulting in reduced ability to absorb, transport and store vitamin A.

However, toxicity and metabolism of PA are markedly different among animal species. Susceptible animals to PA are cattle, horses, rats and chickens, whereas the resistant species include gerbils, hamsters, guinea pigs, sheep and Japanese quail (Cheeke and Shull, 1985). Susceptible species such as rats, cattle and horses have a high rate of pyrrole production, whereas PA-resistant animals such as sheep and Japanese quail have a low pyrrole production rate (Cheeke and Shull, 1985; White et al., 1973). Moreover, adding sulfur amino acids such as cysteine and synthetic antioxidants such as ethoxyquin and butylated hydroxyanisole (BHA) to the diet had a pronounced protective effect against PA toxicity in rats (Buckmaster et al., 1976) and mice (Mirranda et al., 1981a, 1981b), but not effect in chickens (Dickinson and Ball, 1985) and beef cattle (Cheeke et al., 1985). Different species may have different pathways for PA metabolism. For example, sheep accumulate toxic levels of copper in the liver when consuming PAs, yet they are very resistant to PA toxicosis.

The objectives of this study were to: 1) determine if hepatotoxic effects are necessary

to influence copper and vitamin A metabolism, and 2) study the interactions in hepatotoxic PA present in tansy ragwort (*Senecio jacobaea*), copper and vitamin A metabolism in chickens and Japanese quail.



## **Chapter 1**

# **LITERATURE   REVIEW**

## 1.1 Liver Histology

The liver contains, in addition to its parenchymal cells (hepatocytes), several types of nonparenchymal cells that line the sinusoids. The main nonparenchymal cells are the endothelial cells, the kupffer cells and the stellate cells.

*Parenchymal cells (hepatocytes)* make up about 90% of the total liver mass, and represent about 65% of the total liver cells. These cells have the widest profile of enzymes, which permit a wide variety of biochemical reactions, of all tissue cells in the body. They have three functional surfaces: the most extensive surface is adjacent to the sinusoids and is covered by microvilli; the contact surface between adjacent liver cells is smooth; the small surface forming the bile canaliculus is set off by desmosomes with components (Dellmann and Brown, 1976). These cells are considered the main uptake site of vitamin A (Sporn et al., 1984).

*Endothelial cells* are a barrier between the sinusoidal blood and the parenchymal cells and have many pores. These fenestrations, which are grouped together as so-called "sieveplates", influence the filtration of particles from blood to the parenchymal cells and vice versa (Wisse and Knook, 1979). The endothelial cells have an important function in the clearance and degradation of denatured protein and lysosomal enzymes.

*Kupffer cells* are the largest group of tissue macrophages in mammals. A large part of the cell surface is exposed to the blood flowing through the liver (Figure 1.1). A main function of kupffer cells is to phagocytose foreign particles, such as bacteria or colloids (Wisse and Knook, 1979). They may be involved in the uptake of particulate or soluble

immune complexes (Skogh et al., 1985).

***Stellate cells*** are localized within the space of Disse (figure 1.1.) These cells have been designated by a variety of names such as pericytes, fat-storing cells, interstitial cells, lipocytes, Ito cells, lipid-storing cells and vitamin A-storing cells. Stellate cells may have a role in the synthesis of connective tissue compounds, and may also be involved in the pathologic changes observed during the development of liver fibrosis (Wake, 1980). The cytoplasm of stellate cells contains lipid droplets that alter in size and number with dietary vitamin A status (Moran, 1982). These cells are the main vitamin A storage site in the liver, and they are also found in the intestine, kidney, heart, large blood vessels and testes (Wake, 1980).

***Liver lobule*** is a functional unit of liver tissue (Figure 1.2). It is composed of a portal triad (which consists of a branch of the hepatic artery, a branch of the portal vein and a bile ductule), a central vein, parenchymal cells, sinusoids and bile canaliculus. Blood flows into the sinusoids from portal triads to the central vein and bile flows through the bile canaliculus in the opposite direction into the bile ductules which empty into bile ducts. The region surrounding the portal triads is called zone 1. The cells in this area are rich in oxidative enzymes used in carbohydrate metabolism. The cells surrounding the central veins are considered to make up the zone 3 cells. These cells, on the other hand, are rich in microsomal pathway enzymes (P450 enzymes). Therefore, toxins that are bioactivated by the P450 system would exert their necrotic effects more in zone 3 (centrilobular necrosis) than zone 1. The region between zone 1 and 3 is zone 2. These cells have enzyme profiles ranging from more oxidative to more microsomal depending

on the zone to which they are closer.

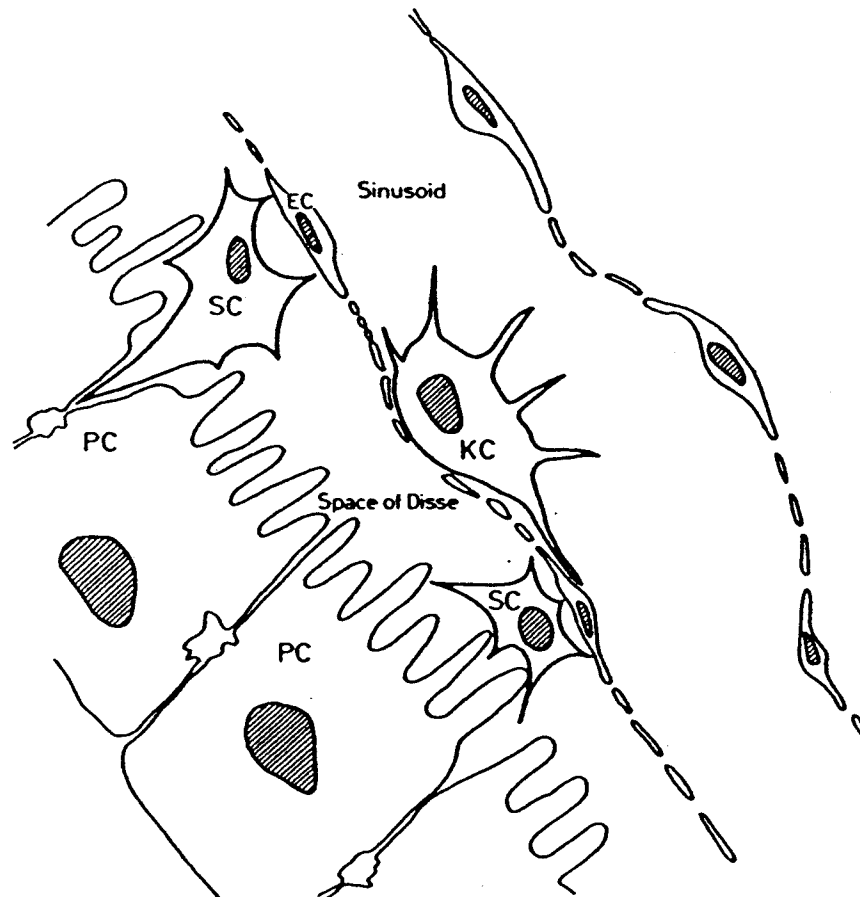


Figure 1.1 Diagram of liver structure. PC (parenchymal cells), EC (endothelial cells), KC (kupffer cells), SC (stellate cells).

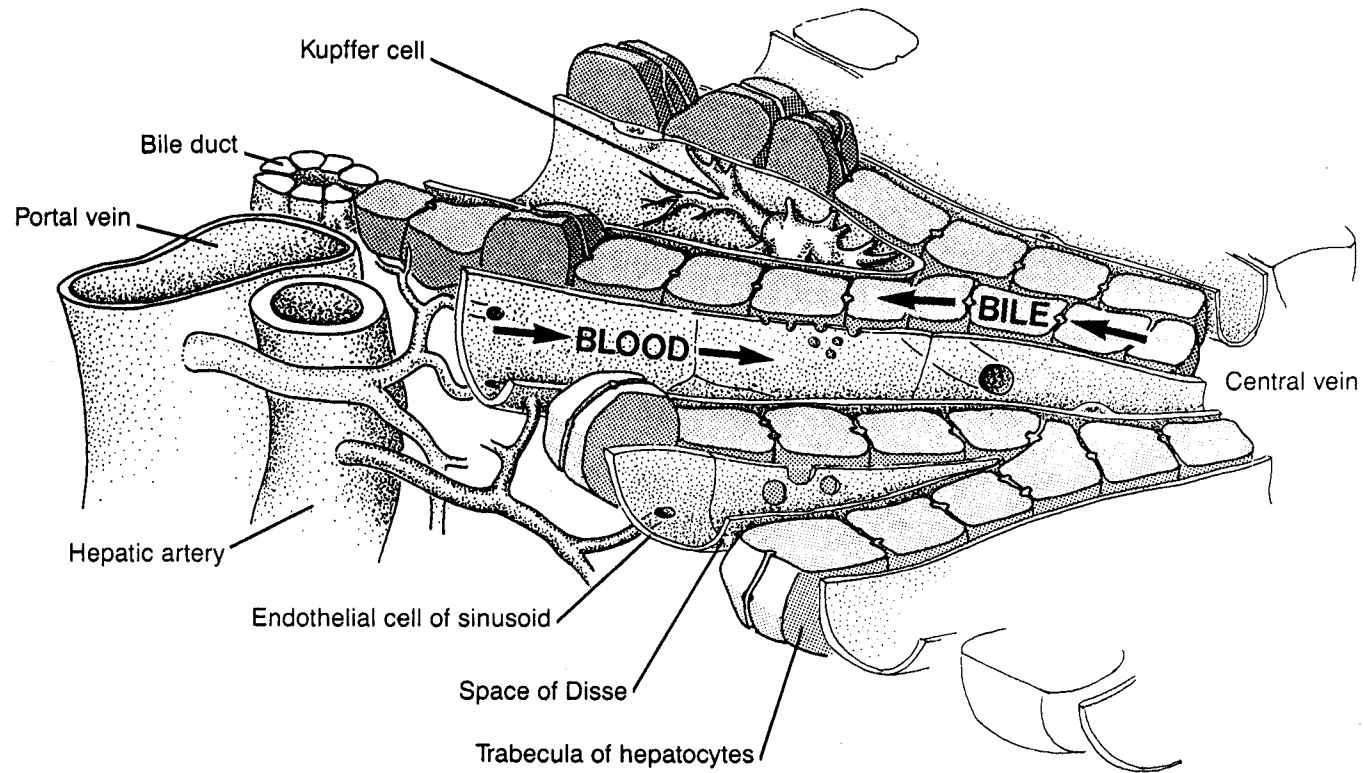


Figure 1.2 Diagram of liver lobule

(From Cormack, 1984)

## 1.2 Vitamin A Metabolism

Vitamin A was the first fat-soluble vitamin to be discovered at the beginning of this century and since then has been a subject of intense research activity. The participation of this important compound in many life processes is well documented. It is required for vision, growth, reproduction, cellular differentiation and proliferation, and the immunity system (Sporn et al., 1984). Since carcinogenesis is a disorder in cell growth and differentiation, it is postulated that vitamin A has a role in cancer prevention and control (Ong and Chytil, 1983). Despite its early discovery, the molecular mechanisms underlying the biological effects of vitamin A are still poorly understood.

In human nutrition vitamin A is one of the few vitamins for which both deficiency and excess constitute serious health hazards. Deficiency occurs in endemic proportions in many developing countries and is considered to be the most common cause of blindness in young children throughout the world. Toxicity usually arises from abuse of vitamin supplementation. In animals, however, these conditions arise as a result of under or over supplementation of vitamin A of animals fed manufactured feeds (McDowell, 1989).

### Chemistry of vitamin A

Vitamin A is a fat soluble, unsaturated 20 carbon cyclic alcohol (Figure 1.3.) containing a  $\beta$ -ionone ring and an unsaturated side chain. Vitamin A has different isomeric forms with different biological activities. All trans-retinol (vitamin A) is the most active and abundant form. Retinal, which is the vitamin A derivative with a terminal aldehyde, is the active form that functions in the visual cycle (Madani, 1986);

retinoic acid, which has a terminal carboxyl group, is a normal, physiological metabolite *in vivo* of retinol, and supports all biological activities of vitamin A excepted vision and reproduction (Bondi and Sklan, 1984). Whereas retinol and retinal can be reversibly oxidized and reduced in the body the further oxidation of either of these two forms to retinoic acid is irreversible. Retinyl esters, which are predominately retinyl palmitates, are the storage forms of vitamin A in the body (Madani, 1986).

### **Natural sources of vitamin A**

The primary source of vitamin A to the animal kingdom is from plant supplies, primarily as the provitamin A carotenoids. In addition to these sources, vitamin A is available as the retinyl esters from animal sources. Carotenes, mainly  $\beta$ -carotene in fresh green crops, hays and silages are the most common dietary sources of vitamin A in livestock. The concentration of carotene in green crops fluctuates between 200 to 800 mg/kg dry matter (Bondi and Sklan, 1984). The content of various species of green forages varies widely and is strongly influenced by conditions of growth and the age of the plants. Retinol and its ester-forms are available in animal products like liver, eggs, milk, meat, and oils from the livers of cod fish, shark and whale. Absorption of  $\beta$ -carotene and retinyl esters depend on adequate fat being present in the gut, as well as the presence of bile acids. Absorption efficiency of these compounds decreases as the dietary intake increases (Bondi and Sklan, 1984). However, utilization of  $\beta$ -carotene varies considerably with pronounced species differences (Table 1.1). Chickens and rats have a high conversion efficiency, while humans, cattle, and sheep have a low conversion efficiency (Bondi and Sklan, 1984). Conversion efficiency decreases with increasing

carotene intake. For example, in sheep conversion efficiency decreases from 25% at a  $\beta$ -carotene intake of 20  $\mu\text{g/kg}$  to 7% at an intake of 160  $\mu\text{g/kg}$  (Ullrey, 1972).

Retinol, retinyl esters and  $\beta$ -carotene are all very unstable in the presence of oxygen, moisture, heat and UV light. Other factors such as trace metals and hydroperoxides of fatty acids present in the feed have deleterious effects on vitamin A and  $\beta$ -carotene stability. Under these conditions, oxidation of these compounds which leads to loss of biological activity is likely to occur (Bondi and Sklan, 1984).

### **Intestinal absorption of vitamin A**

The main sources of vitamin A in the diet are provitamin A carotenoids from vegetables and retinyl esters from animal tissues. The retinyl esters are hydrolysed in the intestinal lumen by pancreatic hydrolases, and become components of lipid micelles. Retinol diffuses from the micelles into the enterocytes of the intestinal mucosa (Sporn et al., 1984). Bile acids are essential for this process, as well as for solubilisation and uptake of carotene by the micelles and its subsequent absorption (Hollander, 1980). Carotenoids are cleaved by dioxygenase to retinaldehyde, which is then reduced to retinol in the enterocytes (Sporn et al., 1984).

In the enterocytes retinol reacts with long-chain fatty acids to form retinyl esters. The two enzymes that seem to be involved in the intestinal esterification of retinol are acyl coenzyme A:retinol acyltransferase (ARAT) (Helgerud et al., 1982) and a lecithin:retinol acyltransferase (LRAT) (Ong et al., 1987). It seems that LRAT esterifies retinol during absorption of a "normal" load of retinol, and ARAT esterifies excess retinol (perhaps for temporary storage) when large doses are absorbed and intestinal intracellular retinol-



binding protein (CRBP(II) becomes saturated (Blomhoff et al., 1990). The retinyl esters are then incorporated, together with other lipids and with apolipoproteins, into the chylomicrons that are secreted from the enterocytes into the intestinal lymph (Sporn et al., 1984) and move into the general circulation where several processes, such as triacylglycerol hydrolysis and apolipoprotein exchange, result in the formation of chylomicron remnants. Chylomicron remnants, which contain almost all of the absorbed retinol in the form of retinyl esters, enter the plasma compartment, mainly via the thoracic duct and are ultimately cleared by the liver (Sporn et al. 1984; Blomhoff et al., 1984).

#### **Hepatic uptake, storage and mobilization of retinol**

Newly absorbed retinyl esters are largely taken up from the circulation by the liver, mainly in association with the uptake of chylomicron remnants. In the liver, parenchymal cells (hepatocytes) are responsible for uptake of chylomicron remnant retinyl esters (Blomhoff et al., 1984). The retinyl esters are probably hydrolyzed at the plasma membrane or in early endosomes in the parenchymal cells by a retinyl ester hydrolase (Harrison and Gad et al., 1989). Retinol is subsequently found in endosomes with other ligands that are taken up by receptor-mediated endocytosis, and then it is transferred to the endoplasmic reticulum (Blomhoff et al, 1985), where retinol-binding protein (RBP) is found in high concentration. Binding of retinol to RBP apparently initiates a translocation of retinol-RBP to the Golgi complex, followed by secretion of retinol-RBP from the parenchymal cells (Rask et al., 1983).

In parenchymal cells most of the retinol that is derived from chylomicron remnant

retinyl esters is transferred within 2 to 4 hours to hepatic nonparenchymal stellate cells (Blomhoff et al., 1984). The transfer is quite specific, as other components of chylomicron remnants, such as cholesterol and vitamin D, are not transferred (Blomhoff, 1984). Chylomicron remnant retinyl esters must be hydrolyzed before the retinol is transferred to stellate cells (Blomhoff et al., 1988). It is likely that the transport of retinol from parenchymal to stellate cells is mediated by binding proteins. Blomhoff et al (1985) reported that newly absorbed retinol, which is secreted from the parenchymal cells bound to RBP, may be delivered to stellate cells via RBP receptors. Cellular retinol-binding protein (CRBP) may be also involved in a direct transport route between parenchymal and stellate cells (Blomhoff, 1987).

In mammals, about 50 to 80% of the total body vitamin A is normally stored in liver stellate cells as retinyl esters (Blomhoff et al., 1985). Stellate cells store retinyl esters in large cytoplasmic lipid droplets, the size and number of which depend on amounts of vitamin A present (Wake, 1980). The concentration of vitamin A varies in different parts of the liver. Samples taken from the right lobe are consistently higher in vitamin A than the other sections of the liver (Olson et al., 1979).

Before the retinol is transported in the blood, it must be mobilized from the liver. It is believed that parenchymal cells are the exclusive site of retinol mobilization. Retinyl esters are hydrolyzed by retinyl palmitate hydrolase and bind to retinol-binding protein (RBP), a specific binding protein, synthesized in endoplasmic reticulum and then secreted from the cells (Rask et al, 1983). However, a new suggestion is that stellate cells also contain RBP and can mobilize retinol-RBP directly into the plasma without prior transfer

of retinol to the hepatocytes (Blomhoff, 1990).

### **Transport of vitamin A in plasma**

After vitamin A is mobilized from the liver, it is transported in the plasma as retinol bound to a specific 21kD molecular weight binding protein, which contains one binding site for one molecule of retinol. Most of the retinol-RBP in plasma is reversibly complexed with transthyretin (TTR), a 55kD protein which is synthesized in parenchymal cells (Ganguly, 1989), and is therefore less susceptible to filtration by kidney glomeruli (Sporn et al., 1984). The retinol-RBP-TTR complex is transported to the cells of various tissues, where the complex binds to a cell-surface receptor and retinol then crosses the cell membrane. The affinity of apo-RBP for TTR is less than that of halo-RBP and the apo-RBP dissociates from TTR and is mainly filtered and catabolised by the kidney (Sporn et al., 1984). Most of the plasma retinol that leaves the general circulation is recycled an average of seven to nine times rather than being irreversibly utilized in tissues (Green et al., 1987).

The delivery of retinol to the functional cells may be controlled by processes regulating the synthesis and release of RBP. One such factor is the retinol status of the animal. Soprano et al.(1986) reported that retinol does not control the rate of RBP synthesis or the level of the translatable RBP mRNA in the liver of the rat. In other words, retinol does not control the expression of RBP gene in the liver. However, it was found that RBP accumulated in the liver, particularly in endoplasmic reticulum, and the plasma RBP decreased in vitamin A deficient rats, whereas the liver and serum levels of TTR were not affected by the deficiency and repletion (Dixon and Goodman, 1987). It

confirmed that retinol can affect the secretion of RBP from the cells. Furthermore, protein-energy malnutrition and zinc have been shown to be dietary factors affecting RBP synthesis and liver vitamin A mobilization (Smith et al., 1973; Carney et al., 1976).

### **Cellular uptake and metabolism of vitamin A**

The mechanism for cellular uptake of retinol from plasma is still unclear. It is generally considered that after vitamin A is transported via the plasma into the target cells by binding to the RBP, cellular uptake of retinol is mediated through a specific receptor for retinol-RBP-TTR complex on the cell surface (Sivaprasadarao and Findlay, 1988), where retinol is taken up by the cell and apo-RBP remains outside the cell. In the cell cytosol several similar binding proteins for the physiological transport of retinol analogues have been isolated and identified (Table 1.2). These are cellular retinol binding protein (CRBP), cellular retinoic acid-binding protein (CRABP), cellular retinaldehyde-binding protein (CRALBP) and interstitial retinol-binding protein (IRBP). These cellular retinol binding proteins have a high degree of affinity for their respective retinol analogues (Ong, 1985). After cellular uptake, retinol can be irreversibly oxidized to retinoic acid by alcohol dehydrogenase, whereas retinol is reversibly converted to retinal in cell cytosol (Posch et al, 1989). Both the retinol and retinoic acid can be converted to their respective glucuronides in the presence of UDP-glucuronic acid by the action of microsome glucuronyl transferase (Bondi and Sklan, 1984). These glucuronides which are water soluble constitute a large portion of the retinol excreted through the bile. Esterification and hydrolysis of retinol have been observed in different cells (Bondi and Sklan, 1984).

## **Physiological functions of vitamin A and deficiency effects**

### **1) Vision**

Retinol in the eye is involved in the opsin/rhodopsin visual cycle and maintains the structure of the visual cells as well as their constant regeneration (Ganguly, 1989). After the all-*trans* retinol is delivered to retinal pigment epithelium, it is isomerized by retinol isomericase and then transported by the IRBP to the retina. There, it is oxidized to 11-*cis*-retinaldehyde by the membrane-bound retinol dehydrogenase of the rod outer segment, and the 11-*cis*-aldehyde eventually binds to the protein opsin giving rhodopsin. On exposure to light the *cis*-retinaldehyde of the rhodopsin is converted to the all-*trans* form, and after it is hydrolyzed to the free state it is reduced to all-*trans* retinol by the retinal dehydrogenase associated with the rod outer segment. The electric impulses derived from this reaction are transported to the brain via the optic nerve and recorded in various intensities depending on the amount of light entering the eye (McDowell, 1989). In vitamin A deficiency, the outer segment of the rods lose their opsin, leading to their eventual degeneration. The entire structure becomes filled with the tubules and vesicles, while at the same time the outer segment loses its usual shape and becomes spherical (McDowell, 1989).

### **2) Maintenance of normal epithelium**

Vitamin A deficient animals develop a keratinizing metaplasia in mucus-secreting epithelial cells (Sporn et al, 1984). Tissues that are particularly sensitive to vitamin A deficiency are the trachea, the skin, the salivary glands, the cornea and the testes. There is evidence that vitamin A is necessary for the formation of large molecules containing

glucosamine (Sporn et al., 1984). These are the mucopolysaccharides occurring principally in the mucus secreting epithelia and in the extracellular matrix of cartilage, mainly as chondroitin sulfate (Bondi and Sklan, 1984). Vitamin A has a role in affecting the biosynthesis of all types of glycoconjugates including glycoproteins, glycolipids and proteoglycans (Deluca and Wolf, 1972). In the absence of vitamin A, cell surface glycoproteins were found to be under-glycosylated and glycolipids were also affected (Patt et al, 1978).

### **3) Cell differentiation and gene expression**

Epithelial cells from deficient animals fail to differentiate to mucus-secreting cells and mesenchymal cells fail to differentiate beyond the blast stage (Sporn et al., 1984). Green and Watt (1980), using human keratinocyte cells, claimed that the expression of differentiation in keratinizing stratified squamous epithelias is enhanced in vitamin A deficiency. Sato et al (1980) showed also that retinoic acid can block terminal differentiation into mature adipocytes in different cloned lines of murine preadipocytes. Although the effect of vitamin A in cell differentiation has been recognized for many years, the mechanism is still poorly understood. The early studies (Zachman, 1967) showed that vitamin A can stimulate RNA synthesis in the colon and intestinal mucosa of rats. Deluca et al (1971) had produced similar evidence in support of this claim, and reported that the synthesis of rRNA and tRNA in the intestinal mucosa of rats is depressed on vitamin A deficiency. Further work showed that the depressed RNA synthesis in nuclei was due to an effect on the elongation of the nascent RNA chains (Tsai and Chytil, 1978). Recently, three nuclear receptors, which are  $\alpha$ ,  $\beta$ ,  $\gamma$ -retinoic acid

receptors (RARs), have been cloned (Petkovich et al., 1987; Brand et al., 1988; Krust et al., 1989; Zelents et al., 1989), and various retinoic acid response elements (RAREs) and genes that are directly regulated by retinoic acid receptors have been identified (Thé et al., 1990). A suggested mechanism for vitamin A regulation of gene expression proposes that after cellular uptake, retinol can be oxidized to retinoic acid and can then diffuse into the nucleus where it can bind to one of its nuclear receptors. All the nuclear RARs are ligand-dependent transcription factors; that is the retinoic acid-RAR complex regulates gene expression by its interaction with RAREs in the vicinity of target genes (Blomhoff et al., 1990). This hypothesis may also explain the multiple functions of vitamin A in humans and animals.

#### **4) Immune system**

Vitamin A deficiency has long been associated with increased susceptibility to infection by bacteria, viruses and other microorganisms (Sporn et al., 1984). In the humoral immune system, it has been reported that the production of antibodies like IgM and IgG decreases significantly in vitamin A deficient mice (Smith and Hayes, 1987). In the cellular immune system, T lymphocyte populations, natural killer cell activity and mitogen response is significantly decreased in vitamin A deficiency (Smith et al, 1987; Bowman et al., 1990). Furthermore, Bowman et al (1990) showed that the production of lymphokines ( $\gamma$ -interferon), which are secreted from T helper cells, was significantly decreased in vitamin A deficient rats. Since lymphokines have an important role in regulation of both T cells and B cells (Herberman et al., 1979), it appears that vitamin A may impair both humoral and cellular immunity.

## **Dietary factors affecting vitamin A metabolism**

### **1) Protein and fat**

Adequate dietary protein is required in order to synthesize the proteins connected with both the absorption and transport of carotenes and vitamin A. Under conditions of inadequate protein intake, both conversion of  $\beta$ -carotene to retinol in the intestinal mucosa and retinyl ester hydrolysis in the intestinal lumen are depressed (Stoecker and Arnich, 1973; Ascarelli, 1969). The levels of RBP in the serum have been shown to be sensitive to inadequate protein intake (Golden, 1982). Vitamin A uptake and absorption in the intestine is dependent on the presence of lipids and bile acids to form micelles. Thus, disorders of bile or pancreatic secretion reduce the uptake of vitamin A (Sporn et al., 1984).

### **2) Vitamin E**

Vitamin E and other antioxidants present in the diet may protect the sensitive conjugated double bond system of vitamin A from oxidation (Sporn et al., 1984). It was suggested that vitamin E exerts an apparent sparing effect on vitamin A by raising the liver stores and decreasing turnover rate of vitamin A (Jenkins and Mitchell, 1975). High levels of vitamin E in the diet alleviated signs of hypervitaminosis A in chicks (McCuaig and Motzok, 1970) and rats (Jenkins and Mitchell, 1975). However, another report (Frigg and Broz, 1984) showed that various vitamin E intakes had no influence on plasma retinol levels. In contrast, the levels of vitamin E in plasma were markedly decreased by the higher vitamin A supplementation.

### **3) Zinc**

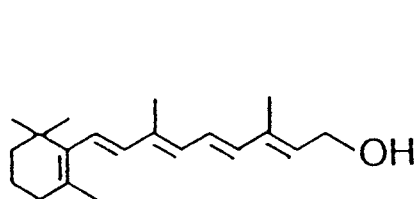
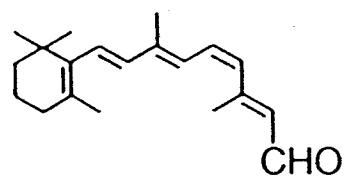
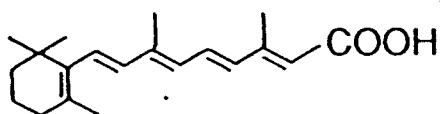
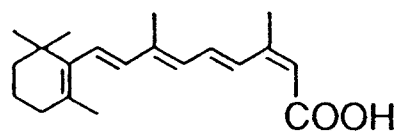
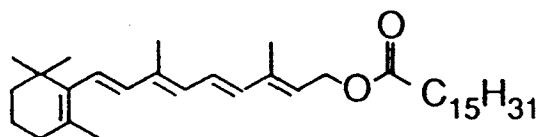


Zinc deficient animals have lower blood retinol and RBP and higher liver vitamin A than zinc adequate animals, even when they received adequate amounts of vitamin A; the plasma vitamin A levels, however, can be restored to normal levels in the same rats by zinc therapy (Smith et al., 1973; Solomons and Russell, 1980). This has been suggested to be zinc involvement in production of RBP and also hydrolysis of retinyl palmitate to retinol in the liver. Since alcohol dehydrogenase is a zinc metalloenzyme, it has been proposed that the effect of zinc deficiency may partly be responsible for the altered vitamin A metabolism (Bondi and Sklan, 1984).

#### 4) Copper

Moore et al (1972) noted that while copper and vitamin A behave similarly in being stored preferentially in the liver and being transported by blood proteins, the tissue concentrations of these nutrients often behave in an inverse manner. Factors that increase the concentration of one usually decrease the concentration of other. Both copper and zinc have been observed in a high molecular weight lipid- protein aggregate (LPA) in liver cytosol. This aggregate contains proteins involved in vitamin A metabolism (Sklan, 1983) including intracellular retinol binding protein, retinyl palmitate and triolein hydrolase, and carotene cleavage activity; the latter are Cu-Zn metalloproteins. Moreover, Rechman et al (1987) reported that in copper deficient rats, retinol and retinyl esters increase significantly in the liver and decrease in the serum. This has been interpreted to mean that copper has an involvement in synthesis of vitamin A transport proteins. However, vitamin A has also a role in the regulation of ceruloplasmin synthesis (Barber and Cousins, 1987). That high levels of vitamin A in the diet result in increasing

copper concentration in the liver and decreasing copper concentration in serum has been reported (Sklan et al., 1987).

all-*trans*-retinol11-*cis*-retinalall-*trans*-retinoic acid13-*cis*-retinoic acid

retinyl palmitate

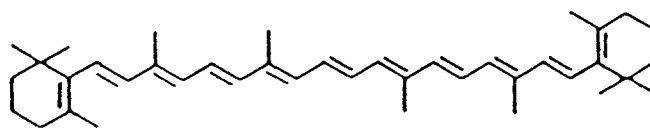
 $\beta$ -carotene

Figure 1.3 Structural formulas of  $\beta$ -carotene and  
some naturally occurring retinoids

Table 1.1 Efficiency of conversion of carotene to vitamin A in different species

Species	Conversion efficiency (%)	IU vitamin A equivalent to 1 mg $\beta$ -carotene
Rat	100*	1667
Chicken	100	1667
Pig	30	500
Cattle	24	400
Sheep	30	500
Horse	33	555
Man	33	555
Dog	67	1111

\* Efficiency of conversion has been set at 100% for the rat.

From Bondi and Sklan, 1984

Table 1.2 Retinoid-binding proteins

Proteins	Molecular weight (kD)	Endogenous ligand	Suggested functions
RBP	21	retinol	blood plasma transport
CRBP	15	retinol	intercellular transport
CRABP	15	retinoic acid	intercellular transport
CRALBP	33	retinal	enzymatic reactions in visual cycle
IRBP	144	retinol & retinal	intercellular transport in visual cycle
CRBP(II)	16	retinol	intercellular transport in small intestine

From Ong, 1985 and Blomhoff et al., 1990

### 1.3 Pyrrolizidine Alkaloids

The pyrrolizidine alkaloids (PA) are a large class of naturally occurring toxicants that are produced by numerous plant species worldwide. As many as 6000 plant species in the world have been estimated to contain PA (Smith and Culvenor, 1981); these plants have been shown to produce toxic effects in grazing animals, rodents, poultry and humans (Cheeke, 1990; Mattocks, 1986; Cheeke and Shull, 1985). Most PA-containing plants that have been implicated in livestock poisoning are in genera *Senecio*, *Crotalaria*, *Heliotropium* and *Echium*.

#### Chemical structure and metabolism of PAs

The PA constitute a large group of alkaloids containing the pyrrolizidine nucleus. Most PA are esters of hydroxylated 1-methylpyrrolizidine. The amino alcohols are called necine, and the acid moieties necic acids (Mattocks, 1986). PA toxicity is influenced by chemical structure. Most cytotoxic PA are esters of the necine base retronecine and heliotridine (Figure 1.4) which are diastereomers with opposite configurations at C7. For hepatotoxicity, there must be a 1,2-double bond and a branch in an esterified side chain. Cyclic diesters are the most toxic, noncyclic diesters are of intermediate toxicity and monoesters are the least toxic (Mattocks, 1986).

The main metabolic routes (Figure 1.5) for PA have been established in laboratory animals: dehydrogenation to pyrrolic derivatives, conversion to N-oxides and hydrolysis (Mattocks, 1986). Dehydrogenation of PA to yield pyrrolic derivatives is the classic pathway of PA metabolism. The parent PA are nontoxic, but mixed function oxidase

(MFO) system enzymes in the liver dehydrogenate the 1,2-dehydropyrrolizidine ring, forming pyrrolic (dehydropyrrolizidine) metabolites that are highly reactive and strong alkylating agents, which cross-link strands of DNA and thus impair cell division. Hydrolysis of the ester groups by esterases with excretion of the acid and amino alcohol products and formation and excretion of highly water soluble N-oxides are thought to represent detoxification mechanisms (Mattocks 1986). One of the ultimate metabolites may be trans-4-hydroxy-2-hexenal (t-4HH), a very reactive aldehyde, identified by Segall et al (1985). This strong oxidant can cause lipid peroxidation resulting in membrane damage and selectively inhibit microsomal enzymes (*in vitro*) such as aminopyrine demethylase, cytochrome P-450 and glucose-6-phosphatase (Segall et al, 1985). Recently, Griffin and Segall (1989) reported that lipid peroxidation may be a secondary effect of PA. They suggested that the primary mechanism of PA for hepatotoxicity may be to inhibit the sequestration of  $\text{Ca}^{++}$  in extramitochondrial and mitochondrial compartments, possibly by inactivating free sulfhydryl groups and oxidizing pyridine nucleotide respectively. They also reported that the metabolites of PA caused markedly decreased cellular glutathione, ATP and NADPH levels and increased  $\text{NADP}^+$  levels in hepatocytes (Griffin and Segall, 1987).

### **Toxicology of pyrrolizidine alkaloids**

General characteristic signs of PA toxicosis include<sup>de</sup> swelling of hepatocytes, centrilobular necrosis, megalocytosis of the parenchymal cells, karyomegaly, progressive fibrosis, bile duct proliferation, veno-occlusion and loss of hepatic metabolic functions. Other common clinical signs of PA toxicity are rough unkempt appearance, diarrhea,

prolapsed rectum, ascites, edema of tissues of the digestive tract, lassitude and dullness, photosensitization reactions and abnormal behavior (Cheeke and Shull, 1985).

### **1) Species differences in PA toxicity**

Similar hepatotoxic effects of PA have been demonstrated in several animal species but major differences exist in species susceptibility to PA poisoning. Among animal species, sheep, goats, guinea pigs, rabbits and hamsters are highly resistant to PA whereas rats, mice, cattle and horses are highly susceptible. Chickens and turkeys are susceptible to PA toxicosis (Cheeke and Pierson-Goeger, 1983) but Japanese quail are resistant to a tansy ragwort cumulative intake of several thousand percent of body weight (Table 1.3) (Buckmaster et al, 1977).

The major determinant of susceptibility to PA toxicity appears to be the extent by which the different species convert PA to pyrrolic metabolites through the action of hepatic microsomal enzymes. Several research results showed those susceptible species such as rats, cattle and horses had a high rate of pyrrole production whereas PA-resistant animals such as sheep and Japanese quail had low pyrrole production (Shull et al, 1976). In some cases, however, as for example with rabbits, the in vitro pyrrole production rate is high (Shull et al., 1976; Pierson et al., 1977). Their resistance might be due to an ability to rapidly detoxify pyrroles, a suggestion supported by a high rate of urinary excretion of PA metabolites in the rabbit (Swick et al., 1982a). A complete understanding of the mechanisms of resistance will require further research on the specific pathways of PA metabolism, which may vary among species (Cheeke, 1988).



## 2) PA toxicity in livestock

Most PA-containing plants that have been implicated in livestock poisoning are in genera *Senecio*, *Crotalaria*, *Heliotropium* and *Echium*. In the early 1900's, an outbreak of livestock poisoning in Nova Scotia was called Pictou disease while in New Zealand a similar problem was called Winton disease. They were both identified to be due to *Senecio* poisoning. Meanwhile, walking disease of horses in parts of U.S., which was a considerable economic problem, was caused by *Senecio* poisoning (Cheeke and Shull, 1985). In the 1960s, *S. jacobaea* (tansy ragwort) became a significant problem in the U.S. Pacific northwest. Several million dollars of livestock losses have occurred since that time (Cheeke and Shull, 1985). The symptoms of livestock poisoning including liver cirrhosis, jaundice, and in the case of horses, staggering, pressing the head against objects, and walking in a straight line often can be seen. Death is always caused by irreversible liver damage (Cheeke and Shull, 1985).

## 3) Human exposure to PA

The main routes of human exposure of PA include consumption of foodstuffs contaminated with PA-containing plants, herb tea, herbal remedies, and animal products such as milk, meat, eggs and honey from animals exposed to PA (Huxtable, 1989).

The major toxic action of PA in humans are hepatic veno-occlusive disease, pulmonary arterial hypertension, cor pulmonale and carcinogenesis (Huxtable, 1989). Two major outbreaks of human veno-occlusive disease that occurred in the period from 1974-1976 in northwestern Afghanistan and central India were due to the consumption of food contaminated *Heliotropium* spp. and *Crotalaria* spp., respectively (Tandon et al., 1978;

Datta et al., 1978). PA-containing plants such as coltsfoot and comfrey which were identified to be carcinogenic in rats have been used in Japan, Australia, Europe and U.S. as vegetables and herb teas for many years (Hirono, 1986). Small amounts of PA have been detected in the milk of animals fed PA containing plants. Dickinson et al.(1976) administered tansy ragwort via rumen canula to lactating cows, and found that the milk contained 0.5-0.8 ppm PA. Goeger et al (1982a, 1982b) fed a diet containing 25% *S.jacobaea* to dairy goats, and detected 7.5 ppm of PA (dry weight basis) in the milk. When the milk was fed to rats, the mild liver lesions were found. Furthermore, Deinzer et al (1977) examined several honey samples obtained from regions in Oregon and Washington states; levels of 0.3-3.9 ppm PA were detected. Similar results were reported by Culvenor et al (1981); honey from bees foraging on *Echium* spp. in Australia contained 0.27-0.95 ppm of PA.

#### **4) PA toxicity and mineral metabolism**

Bull et al.(1956) reported that sheep with chronic consumption of *Echium* and *Heliotropium* had elevated levels of liver copper and developed chronic Cu toxicosis. Since then, other studies of PA effects on mineral metabolism have been reported. PA exposure results in elevated liver copper and iron and decreased zinc in horses (Garrett et al., 1984), rabbits (Swick et al., 1982a) and rats (Swick et al, 1982b, 1982c). The mechanisms by which exposure to PA causes excessive hepatic copper levels are still not completely understood. There may be a breed or species difference in susceptibility. Culvenor et al.(1984) fed *E. plantagineum* to merino sheep and found only slight hepatotoxic effects and no elevation in liver copper concentrations, while Seaman reported

(1987) a field outbreak of chronic copper poisoning in British breeds of sheep grazing *E. plantagineum*. Swick et al.(1983) and White et al.(1984) observed no increase in liver copper levels in lambs suffering from chronic *Senecio jacobaea* toxicity. Liver copper concentrations were normal in cattle with chronic heliotrope poisoning (Bull et al., 1961). Horses and rabbits consuming *S.jacobaea* had liver copper levels almost 6 and 2.6 fold high than controls, respectively (Garrett et al., 1984; Swick et al., 1982a).

The levels of copper accumulation in the liver may be correlated with both the intakes of copper and PA. Short-term, acute PA exposure is less likely than chronic exposure to result in copper accumulation (Cheeke, 1989). Swick et al. (1982b) found that accumulation of liver copper in rats fed *S. jacobaea* occurred only in the presence of high (250 ppm) copper levels, and with low PA intakes. Similar results have been seen in Australia in sheep fed heliotrope; elevated liver copper levels occurred only when copper was also administered (Howell et al., 1988).

In chronic copper toxicity, copper is initially accumulated in all subcellular fractions and then concentrated in the nuclei and debris (Helman, 1983; Gooneratne, 1979). As these fractions become saturated with copper, the lysosomes take up the element. Because of their increased density they then separate with the nuclei and debris fraction during centrifugation. Rupture of the lysosomes *in vivo* releases hydrolases and the copper, causing cell death and copper-induced hemolysis (Gooneratne et al., 1979).

PA exposure may also influence on the metabolism of other minerals. Rats fed tansy ragwort had reduced liver and serum zinc levels (Swick et al. 1982b, 1982c). In copper-loaded sheep, the subcellular distribution of zinc is altered, with a decrease in the

proportion in the cytosol fraction (Gooneratne et al., 1979). Possibilities have been suggested to explain these results. Copper has a higher affinity for metallothionein than zinc and may displace it. Zinc might have protective effects against PA toxicosis by "soaking up" pyrroles with metallothionein (Cheeke, 1990). In addition, there is a marked effect of PA on hematopoiesis and iron metabolism. The iron levels were increased in liver and spleen, when rats (Swick et al., 1982b) and monkeys (Van der watt et al., 1972) were fed PA. A likely explanation is that PA may inhibit heme biosynthesis in the liver and other tissues as a result of the alkylation of DNA; then iron cannot be used for hemoglobin formation with the excess accumulated in the liver and spleen (Cheeke, 1989). In addition, PA had no effect on tissue levels of selenium in rats (Shull et al., 1979) and turkey poults (Burguera et al., 1983).

#### **5) PA toxicity and vitamin A metabolism**

PA have a marked effect on vitamin A metabolism. Moghaddam and Cheeke (1989) observed that in rats fed tansy ragwort, both plasma and liver vitamin A levels were markedly depressed. Significant reductions in plasma vitamin A occurred by 10 days after initial PA consumption. The results may indicate that an influence on vitamin A distribution occurs early in PA toxicosis. Vitamin A is mainly stored in stellate cells (Blomhoff, 1987) while PA damage occurs primarily in hepatocytes. Two mechanisms for the depressed liver and plasma vitamin A levels during PA exposure have been suggested (Cheeke, 1990). First, PA may inhibit the synthesis of proteins involved in vitamin A transport and storage. It is likely that synthesis of retinol-binding protein (RBP) and other proteins involved in vitamin A metabolism is impaired. Secondly, PA

may inhibit vitamin A absorption. Biliary hyperplasia and impaired bile secretion are characteristic of PA toxicosis. Bile is necessary for absorption of fat-soluble vitamins; thus depressed tissue vitamin A levels may reflect diminished absorption. Also, PA damage may impair the ability of the small intestine to take up vitamin A. Several intestinal lesions, including inhibition of crypt cell mitosis and villus atrophy occur in PA toxicosis (Hooper, 1975).

In addition, a copper-vitamin A- vitamin E-PA interaction has been suggested (Cheeke, 1990). PA toxicity induced increased copper levels and decreased vitamin A levels in both liver and plasma. Copper can increase lipid peroxidation, which could increase vitamin E requirements and increase vitamin A destruction. Copper has a cofactor role in synthesis of vitamin A transport proteins (Rachman et al., 1987) while vitamin A has a role in the regulation of ceruloplasmin synthesis (Barber and Cousins, 1987). PA metabolites are prooxidants causing peroxidation damage at the subcellular level (Segall et al., 1985), while vitamin E is a tissue antioxidant which could protect against PA damage. Synthetic antioxidants have protective activity against PA toxicosis (Miranda et al., 1981a, 1981b; Garrett et al., 1984). Red blood cells from rats fed tansy ragwort were susceptible to *in vitro* hemolysis (Moghaddam and Cheeke, 1989), suggesting depletion of vitamin E by PA exposure. Both vitamin A and vitamin E metabolism can be affected by PA exposure.

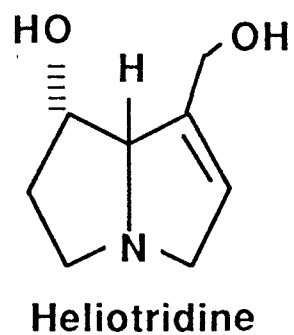
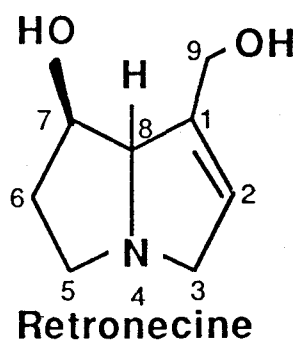


Figure 1.4 Typical PA necine bases

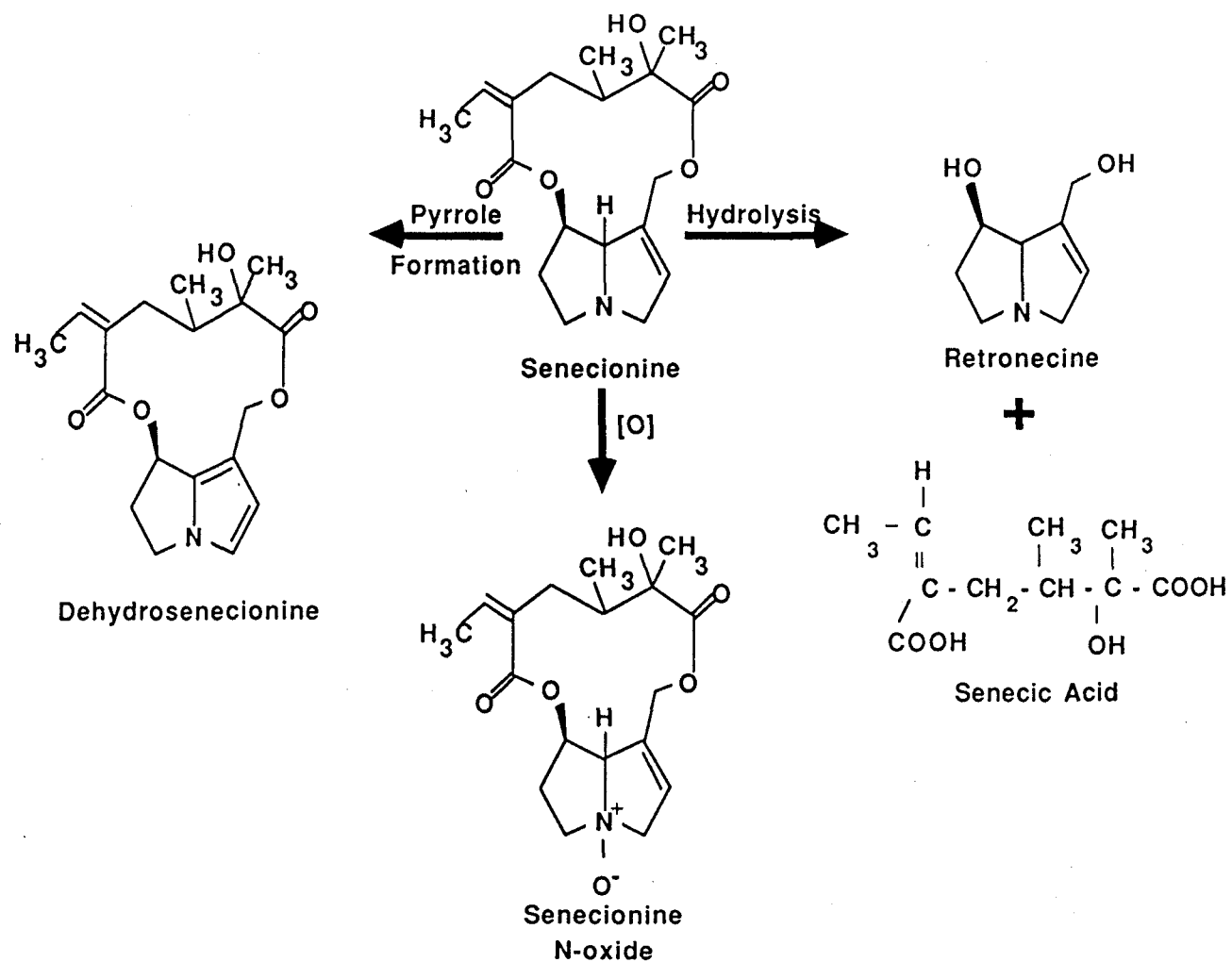


Figure 1.5 Major pathways for the metabolism of the PA senecionine

(From Winter and Segall, 1989)

Table 1.3 Characterization of animal species by susceptibility to pyrrolizidine alkaloid toxicity *in vitro* hepatic pyrrole production rate

Species	Susceptibility to PA toxicosis	<i>In vitro</i> pyrrole production rate	Lethal dose (as % of body weight)
Cow	high	high	3.6
Horse	high	high	7.3
Sheep	low	low	302
Goat	low	?	205
Rat	high	high	21
Mouse	intermediate	high	?
Rabbit	low	high	113
Guinea pig	low	low	119
Hamster	low	high	338
Gerbil	low	?	3640
Chicken	high	low	39
Japanese quail	low	low	2450

From Cheeke and Shull, 1985.



## **Chapter 2**

### **METHODOLOGY**

## 2.1 Introduction

Many different methods are available for determining vitamin A, trace minerals and ceruloplasmin (CP) in serum and liver, and  $\text{Ca}^{++}$  dependent protein (CDP, or calpain) in muscle. The method used for vitamin A determination was based on the methods of (Bieri et al., 1979; Chow et al., 1983; Omaye et al., 1986) as modified by R.R. Lowry (personal communication). The assay used for analysis of copper, zinc and iron of serum and liver followed the AOAC (1980) methods. The selenium analysis procedure was adapted from Brown and Watkinson (1977). The measurement of CP was similar to the Kim et al. (1988) and Schosinsky et al. (1974) methods. The method used on calpain measurement was according to Gopalakrishna et al. (1985), and modified by Bor-rung Ou (personal communication).

## **2.2 Determination of serum and liver retinol (vitamin A) by high performance liquid chromatography (HPLC)**

### **I. Chicken serum**

#### **1) Principle**

A given volume of serum is diluted with ethanol, which denatures serum proteins, and the vitamin A is extracted with a suitable organic solvent. After centrifugation, an aliquot of the organic phase is injected onto a reversed phase HPLC column, followed by an eluting solvent of suitable polarity. Retinol, which is eluted as a sharp peak within about 10 minutes, is detected by a sensitive UV detector set at 292 nm. Retinol is quantitated by use of peak height ratios relative to an internal standard (all *trans*-retinol acetate).

#### **Apparatus**

- 1) Analytical microbalance (Perkin-Elmer AD-2)
- 2) Centrifuge (IEC HN-SII with 809 head, Damon/IEC Division)
- 3) Vortex mixer (VWR model K-550-G)
- 4) Hot plate (at 50°C, with tube block and nitrogen jets)
- 5) HPLC (Perkin-Elmer 601) with a variable wavelength detector (Perkin-Elmer LC-55 spectrophotometer)
- 6) Reversed phase column (C-8, 4.6x250 mm LiChrosorb packed by Brownlee with a 30 mm guard column)
- 7) Polytron (Model PT 10/35, Brinkmann instruments, Inc.).
- 8) Micropipettes (Gilson 100  $\mu$ l, 500  $\mu$ l, 1000  $\mu$ l).
- 9) Syringes (Hamilton, Inc. 10  $\mu$ l, 25  $\mu$ l; Unimetrics Corp. 100  $\mu$ l)

10) Amber glass screw cap culture tubes (16x100 mm)

11) Nitrogen

### Reagents

1) All *trans*-retinol acetate (highest purity, Sigma)

2) Butylated hydroxy toluene (BHT) (Eastman)

3) Heptane (99+ % GC, Eastman)

4) Ethanol (AR reagent, MCP)

5) Methanol (AR reagent, Mallinckrodt)

6) Hexane (99+ % GC, glass redistilled 5°F drum stock)

7) Ethyl ether (AR reagent, Mallinckrodt)

8) Potassium hydroxide (KOH) (ACS reagent, Mallinckrodt)

9) Hydrochloric acid (HCl) (ACS reagent, Mallinckrodt)

### Procedures (carried out in subdued light)

1) 500  $\mu$ l of serum was placed in a 16x100 mm amber tube. To it is added 500  $\mu$ l of the combined internal standard (all *trans*-retinol acetate)/anti-oxidant (BHT) ethanol solution and this is vortexed for 20 sec.

2) 1500  $\mu$ l of heptane/BHT solution is then added and vortexed for 60 sec. This is then centrifuged at 600 g. for 3 minutes. 1000  $\mu$ l of upper heptane layer is transferred to a 16x100 mm amber tube.

3) The solvent is evaporated using a stream of nitrogen at 50°C on hot plate, 100  $\mu$ l of methanol is then added and 40  $\mu$ l of solution is injected into the reversed phase column with 100  $\mu$ l syringe.

- 4) The mobile phase methanol:water (93:7, v/v) is pumped at 0.75 ml/min.
- 5) Absorption measurements are made at 292 nm. this wavelength yields sufficient results for both retinol (vitamin A) and  $\alpha$ -tocopherol (vitamin E).

**Calculation formula:**

$$\text{IU/ml (serum)} = (A \times B \times C \times D \times K_e) / (E \times F \times G \times H \times R);$$

A: total internal standard content per injection ( $\mu\text{g}$ )

B: total volume of heptane used ( $\mu\text{l}$ )

C: volume of methanol used ( $\mu\text{l}$ )

D: peak height of injected sample (mm)

$K_e$ : ratio of retinol (g)/ retinol acetate (g)

E: volume of heptane dried after extraction ( $\mu\text{l}$ )

F: volume of methanol injected ( $\mu\text{l}$ )

G: peak height of injected internal standard (mm)

H: volume of sample used (ml)

R: factor  $0.3 \mu\text{g retinol} = 1 \text{ IU}$

**Internal standard concentration:** 0.090 mg of retinol acetate and 125 mg of BHT in 100% ethanol

## **II. Chicken liver**

### **Principle**

A weighed sample of liver is homogenized with a known volume of distilled water. Homogenate is mixed with ethanol, which denatures liver proteins and enzymes. The

mixture is extracted with a suitable organic solvent. After centrifugation, an aliquot of the organic phase is saponified with alkali (KOH) to break the ester bond of retinol palmitate (main form of vitamin A in liver) and remove other lipids such as triglycerides and phospholipids. After esterification, the free retinol is extracted by addition of a suitable organic solvent. An aliquot of the organic solution is injected into a reversed phase HPLC column, followed by an eluting solvent of suitable polarity. Total retinol, which is eluted as a sharp peak within 10 minutes, is detected by a sensitive UV detector set at 292 nm. Retinol is quantitated by use of peak height ratios relative to an internal standard (*all trans*-retinol acetate).

**Apparatus** (same as for serum)

**Reagents** (same as for serum)

**Procedures** (carried out in subdued light)

- 1) 0.5 g frozen liver sample is weighed and put into a 16x100 mm amber tube with 500  $\mu$ l distilled water, and then homogenized with polytron using a PT-10 probe (speed no. 6) for 20 seconds.
- 2) 1000  $\mu$ l methanol containing BHT is added to homogenate and vortexed for 15 sec. This is then mixed and vortexed with 1500  $\mu$ l heptane/ethyl acetate (70:30, v/v) for 30 seconds, this mixture is centrifuged for 3 minutes at 600 g, and 500  $\mu$ l upper portion transferred into a 16x100 mm amber tube.
- 3) The solvent is evaporated with nitrogen at 50°C on hot plate. 1000  $\mu$ l ethyl ether and 1000  $\mu$ l 0.4N KOH in 100% methanol is then added and capped, and the sample transesterified for 30 minutes at 50°C on a hot plate.

- 4) After saponification, 1000  $\mu$ l hexane and 1000  $\mu$ l 0.5N HCl is added and centrifuged for 3 minutes at 600 g.
- 5) The upper organic layer is completely removed into another 16x100 mm amber tube, and 2000  $\mu$ l ethyl ether/hexane (1:1, v/v) is then added and centrifuged for 3 minutes at 600 g.
- 6) The upper layer is completely removed again and combined with the first portion, then evaporated with nitrogen at 50°C on hot plate.
- 7) Methanol containing the internal standard (*all trans*-retinol acetate) is added; an aliquot of solution is injected into reversed phase HPLC column.
- 8) The detector is set at 292 nm, and the mobile phase methanol:water (93:7, v/v) is pumped at 0.75 ml/min.
- 9) Retinol is quantitated by use of peak height ratios relative to an internal standard.

**Calculation formula:**

$$\text{IU/g} = (\text{A} \times \text{B} \times \text{C} \times \text{D} \times \text{E} \times \text{Ke}) / (\text{F} \times \text{G} \times \text{H} \times \text{R} \times \text{W});$$

A: standard solution concentration ( $\mu\text{g}/\mu\text{l}$ )

B: volume of injected standard solution ( $\mu\text{l}$ )

C: peak height of injected sample (mm)

D: total volume of heptane/ethyl acetate ( $\mu\text{l}$ )

E: volume of ethanol containing internal standard used ( $\mu\text{l}$ )

Ke: ratio of retinol (g)/ retinol acetate (g)

F: peak height of injected internal standard (mm)

G: volume of injected sample ( $\mu\text{l}$ )

H: volume of heptane/ethyl acetate dried after extraction ( $\mu$ l)

R: factor  $0.3 \mu\text{g}$  retinol = 1 IU

W: dried sample weight (g)

**Internal standard concentration:** 2.652 mg of retinol acetate and 125 mg of BHT in 100% ethanol.



## **2.3 Assay for serum and liver copper, zinc and iron analysis by atomic absorption spectrophotometer (AAS)**

### **I. Chicken serum**

- 1) 1.0 ml serum samples were diluted with 4.0 ml 0.1 N HNO<sub>3</sub> and mixed on vortex.
- 2) samples were aspirated into atomic absorption spectrophotometer (Perkin-Elmer model 403) equipped with a recorder (Perkin-Elmer model 56).
- 3) The wavelength were selected for copper (324.8 nm), zinc (213.9 nm) and iron (248.3 nm), respectively.
- 4) The ranges of standard concentration were used as 0.05-0.15 ppm for copper, 0.1-0.4 ppm for zinc.
- 5) Appropriate dilutions were made for samples off scale with 0.1 N HNO<sub>3</sub>.
- 6) Standard curve was prepared directly from recorder response signals of standard solutions and was used for determination of samples.

#### **Calculation formula:**

$$\text{PPM } (\mu\text{g/ml serum}) = \text{dilution factor} \times \text{ppm of aspirated sample}$$

### **II. Chicken liver**

- 1) Approximately 1.0 g wet frozen liver was weighed and put into 50 ml oven-dried tared erlenmeyer flasks.
- 2) Samples were dried in an oven for 24 hours at 65°C and then cooled in a dessicator and reweighed.
- 3) 10 ml 15.7 N concentrated reagent grade nitric acid and 2 ml 11.6 N concentrated

reagent grade perchloric acid were added to each sample.

4) Samples were predigested overnight until clear yellow with no undissolved traces.

5) Samples were heated on a hot plate (185°C) until dry with white powder remaining in flask.

6) The residue was dissolved with exactly 5 ml 0.1 N HNO<sub>3</sub>.

7) Samples were aspirated into atomic absorption spectrophotometer (Perkin-Elmer model 403) equipped with a recorder (Perkin-Elmer model 56).

8) The wavelength were selected for copper (324.8 nm), zinc (213.9 nm) and iron (248.3 nm), respectively.

9) The range of standard concentration were used as 0.25-1.0 & 1.0-5.0 ppm for copper, 1.0-4.0 ppm for zinc and 2.5-20 ppm for iron.

10) Appropriate dilutions were made for samples off scale with 0.1 N HNO<sub>3</sub>.

11) Standard curve was prepared directly from recorder response single of standard solutions and was used for determination of samples.

**Calculation formula:**

$$\text{PPM } (\mu\text{g/g dry tissue}) = (\text{dilution factor} \times \text{ppm of aspirated sample})$$

divided by dry weight sample.

## **2.4 Method for tissue selenium analysis**

### **by semi-automated fluorometer**

#### **Chicken liver**

- 1) Approximately 0.5 g wet frozen liver was weighed and put into 50 ml oven-dried tared erhlenmeyer flask.
- 2) Samples were dried in an oven for 24 hours at 65°C and then cooled in a dessicator and reweighed.
- 3) 10 ml 15.7 N concentrated reagent grade nitric acid and 3 ml 11.6 N concentrated perchloric acid were added to the flask and predigested overnight at room temperature.
- 4) Samples were digested on hot plate (185°C) to "white fumes" and digestion continued for 15 minutes.
- 5) 1.0 ml 12.1 N concentrated reagent grade hydrochloric acid was added to the flasks and digested for 15 minutes after the appearance of "white fumes".
- 6) Samples were removed from heat and cooled down.
- 7) 15.0 ml of 0.027 N EDTA and 2 drops of the combination indicator (cresol red & brom cresol green) were added to the samples and shaken.
- 8) Samples were titrated with 5 N NH<sub>4</sub>OH to "yellow" (PH ≈ 2-3), and the flasks were then weighed (final weight).
- 9) Subtract tare weights from final weights (volume).
- 10) Samples were aspirated into Alpkem II semi-automated fluorometer (Alpkem Corp.) equipped with a recorder, and 0.5% 2,3-diaminonaphthalene (DAN), as a fluorescing

agent, in cyclohexane carrier was automatically mixed with standard solution and samples in auto-analyzer manifold.

11) The system used a general purpose mercury lamp with two filters, a primary narrow pass filter that peaked at 360 nm and a secondary filter having maximum transmission at 550 nm.

12) The range of standard concentration was between 0.9 and 13.5 ng/ml selenium.

13) Samples outside the upper limits of the standard range were diluted with re-distilled water that was adjusted to a pH of 2-3.

14) Calculate standard concentration:

total ng Se added (as SeO<sub>3</sub>) (step 3)/ Volume (step 9);

15) Measure peak height of each standard, blank and sample from the chart.

16) Plot peak height vs standard concentrations (ng/ml) and determine the equation for the line using the least squares fitting routine on the calculator.

17) Determine the solution concentration (ng/ml) for the samples using the above equation.

**Calculation formula:**

$$\text{ng Se/g dry tissue (ppb)} = \frac{\text{solution concentration (step 14)} \times \text{volume (step 9)}}{\text{dry weight of tissue (step 2)}}$$

## 2.5 Measurement of ceruloplasmin (CP) activity in plasma by $\sigma$ -dianisidine dihydrochloride

### Reagents

- 1) MES buffer: 0.1 M morpholino-ethane sulfonic acid (PH 6.1)
- 2) ODA reagent: 2.5 mg  $\sigma$ -dianisidine dihydrochloride/ml (w/v)
- 3) 9 M sulfuric acid (9 mol/l)

### Procedure

- 1) Pipet 0.75 ml of MES buffer and 50  $\mu$ l of serum sample into two tubes (one marked '10 min. and the other '60 min.).
- 2) Pre-incubate the tubes at 40°C for 5 min.
- 3) 0.2 ml of ODA reagent (pre-incubated at 40°C) is added into each tube, mixing and starting the timer at the first substrate addition.
- 4) After exactly 10 min. and 60 min., the '10 min. and '60 min. tubes are removed from the water bath respectively, and add 2.0 ml of 9 M sulfuric acid and mix immediately.
- 5) Measure the absorbance ( $A_{10}$ ,  $A_{60}$ ) of solution at 540 nm, in a cuvet having a 1 cm light path vs de-ionized water as a blank.

### Calculation formula:

$$*U/ml = (A_{60} - A_{10}) \times 125 \text{ U/ml};$$

\* The enzymatic activity of ceruloplasmin is expressed in unit, in terms of substrate consumed.

factor = 125 U/ml.

## **2.6 Measurement for tissue calpain activity**

### **by hydrophobic chromatography**

#### **Chicken leg muscle**

1. Homogenize muscle tissue (5 g) in 5 volume of homogenization buffer (50 mM Tris-HCl [pH 7.5], 1 mM EDTA, 10 mM 2-mercaptoethanol and 150 nM pepstatin A).
2. Centrifuge at 13000 x g at 4°C for 30 min.
3. Mix 5 ml of supernatant with 10  $\mu$ l of 1 mM leupeptin, 1 ml of phenyl sepharose and 0.3 ml of 5 M NaCl, and shake for 5 min.
4. Add 0.6 ml of 0.1 M  $\text{CaCl}_2$  and continue shaking for another 10 min.
5. Pour gel suspension into a 0.8 X 4 cm column (Bio-Rad).
6. Wash with 2 ml of Buffer A (20 mM Tris-HCl [pH 7.5], 0.1 mM  $\text{CaCl}_2$ , 10 mM 2-mercaptoethanol and 20  $\mu$ M leupeptin) containing 0.25 M NaCl to remove calpain inhibitor.
7. Wash with 2 ml of Buffer A.
8. Wash with 2 ml of Buffer A without leupeptin.
9. Elute calpain II (m-calpain) with 4 ml of Buffer B (20 mM Tris-HCl [pH 7.5], 1 mM EGTA and 10 mM 2-mercaptoethanol) containing 0.1 M NaCl and collect this fraction for calpain II assay.
10. Mix 3.0 ml of calpain fraction with 1 ml of casein solution (8 mg/ml casein in solution of 100 mM Tris-HCl and 1 mM  $\text{NaN}_3$ ) and 4 mM  $\text{CaCl}_2$ , and incubate at 25°C (RT) for 30 min.

11. Add 1 ml of ice cold 36% TCA and let stand at 4°C for several hr.
12. Centrifuge for 15 min and take 3.2 ml of supernatant into cuvet.
13. Add 0.8 ml of Bradford solution and incubate at room temperature for 10 min.
14. Measure absorbance (595 nm) by spectrophotometer.

**Calculation formula:**

$$*U/g = (A \times F) / \text{tissue wt (g)};$$

\* 1 Unit (U) = the amount of the enzyme which caused an increase of 0.1 absorbance unit at 595 nm after incubating 30 min. at 25°C.

A: the values of absorbance

F: dilution factor = 4.167

## **Chapter 3**

### **EXPERIMENTAL**



### 3.1 Effects of dietary pyrrolizidine alkaloids on copper and vitamin A tissue levels in the chicken

#### Summary

The effect of feeding the pyrrolizidine alkaloid (PA)-containing plant tansy ragwort (TR)(*Senecio jacobaea*) on tissue levels of copper and vitamin A in the chicken was examined. The possible interaction between copper and vitamin A was also investigated. The experiment was a 2x2x2 factorial design with dietary 0 and 5% TR, 0 and 250 ppm copper, and 0 and 25,000 IU/kg diet vitamin A. Body weight gain was reduced ( $P < 0.01$ ) in birds fed TR. Both serum and liver copper concentrations were markedly increased ( $P < 0.01$ ) in the TR-fed group with 250 ppm copper supplement. Zinc concentrations in the serum and liver were significantly decreased ( $P < 0.05$ ) in the TR-fed groups compared to TR-free groups. Liver iron was increased ( $P < 0.05$ ) in the TR-fed birds. There was no significant difference in the liver selenium levels ( $P > 0.05$ ) between control and TR-fed chicks.

The serum vitamin A levels were significantly decreased ( $P < 0.01$ ) in all TR-fed groups. The ranges of decrease were from 62 to 72% in four TR-fed groups. The liver vitamin A concentrations were also significantly decreased ( $P < 0.05$ ) in TR-fed groups without vitamin A supplement. There was no interaction between dietary copper and vitamin A levels and tissue concentration of these nutrients. The effects of PA on liver and blood vitamin A concentration may reflect PA inhibition of synthesis of retinol-

binding proteins, or impaired vitamin A absorption from reduced biliary excretion.

## Introduction

The pyrrolizidine alkaloids (PA) are a large and important family of natural toxicants produced by a variety of plant species. Most PA, which produce toxic effects in livestock and humans, are in the genera *Senecio*, *Crotalaria*, *Heliotropium* and *Echium* (Cheeke and Shull, 1985).

The hepatotoxicity and metabolism routes of PA are well known (Cheeke and Shull, 1985; Mattocks, 1986). They also have an important interaction with nutrient metabolism. An interaction between PA toxicosis and minerals (primarily copper) has been noted. The evidence arose from observations that consumption of *E. plantagineum* and *H. europaeum* by sheep led to excessive liver copper concentrations followed by the hemolytic crisis of copper toxicity (Bull et al., 1956). Since then, it has been reported that PA exposure results in elevated liver copper and iron and decreased zinc in horses (Garrett et al., 1984), rabbits (Swick et al., 1982a) and rats (Swick et al., 1982b, 1982c). However, increased liver copper levels in PA-poisoned animals does not always occur. Swick et al.(1983) and White et al.(1984) did not find elevated liver copper levels in sheep consuming *S. jacobaea*. Liver copper concentrations were normal in cattle with chronic heliotrope poisoning (Bull, 1961). Elevation in liver copper occurs both in species susceptible to PA-induced hepatotoxicity and in resistant species. Elevated liver copper has been reported in PA susceptible species such as horses (Garrett et al., 1984) and rats (Swick et al., 1982b, 1982c), and PA-resistant animals such as sheep (Bull et al., 1956; Seaman 1987) and rabbits (Swick et al., 1982a). However, Swick et al.(1982b)

found that elevated liver copper levels in rats fed *S. jacobaea* occurred only in the presence of high levels of copper (50 and 250 ppm) and with low PA intakes. Similar results have been shown by Howell et al. (1988) who found that heliotrope consumption caused elevated levels of tissue copper in sheep only when copper was also administered. This raises the question as to whether hepatotoxic effects of PAs are necessary to influence copper metabolism. Recently, vitamin A, a nutrient stored and metabolized mainly in the liver, was shown to be affected in PA-poisoned rats (Moghaddam and Cheeke 1989). Both plasma and liver vitamin A levels were markedly depressed during PA exposure. The postulated mechanisms have been suggested to be inhibition of retinol-binding protein synthesis and impairment of vitamin A absorption (Moghaddam and Cheeke, 1989).

In addition, it was noted that the concentration of copper and vitamin A in liver have an inverse relationship. Factors that increase the concentration of one usually decrease the concentration of the other (Moore et al., 1972). Rechman et al.(1987) reported that in copper deficient rats, retinol and retinyl esters increase significantly in the liver and decrease in the serum, while Sklan et al.(1987) claimed that high levels of vitamin A in the diet result in increasing copper concentration in the liver and decreasing copper concentration in the serum.

The objectives of this experiment were 1) to study the effects and interactions of PA on copper and vitamin A metabolism; and 2) to compare the effect of administration of high levels of vitamin A either before or after exposure to PA, to determine if liver damage is associated with PA-induced changes in liver vitamin A and copper levels.

## Materials and Methods

**Animals** The eggs were obtained from the Keith Smith farm, Hot Springs, AR. After hatching, feather sexing and one week pre-experiment, ninety six one-week old male broiler chickens (Peterson x Arbor Acres) were randomly assigned to eight groups (Table 3.1) and housed in electrically heated ventilated wood pens with experimental feed and water available *ad libitum*. They were maintained at 18-22°C under 24 hours artificial light condition. Birds were wing banded and individual body weights were obtained initially and at the end of each week. After 4 weeks, six birds from each group were killed for sample preparation, the rest of them were then given PA-free diet (OSU #1705) containing high levels of vitamin A (25,000 IU/kg) for two more weeks.

**Feed** Eight different feeds were used. The basal diet (95 % of total, OSU #1704) is shown on Table 3.2. The remaining 5% of the diet was supplemented with vitamin A or copper mixed with ground corn or tansy ragwort to bring up the diet to 100%. Tansy ragwort was collected in the bloom stage near Corvallis, Oregon, aired dried, ground through a 1 mm screen in a wiley mill and incorporated into the diet in order to make up 5% of the diet. All *trans* retinol palmitate (Sigma chemical company) was used as the vitamin A supplement. The concentration of this compound in the material obtained was 500,000 IU/g. Cupric sulfate (anhydrous powder, Spectrum chemical, MFG. Corp.) was used as the copper supplement. Table 3.1. contains precise information regarding the vitamin A, copper and tansy ragwort composition of each diet. The data of feed intake was collected at the end of the experiment.

**Sample preparation** At the end of the experiment, six birds from each group were killed with injection of T-61 euthanasia solution (Hoechst-Roussel Agri-Vet. company). A blood sample was immediately taken by cardiac puncture from each bird, and serum was separated in a refrigerated centrifuge (Sorvall RC-3 with HG-4L head) at 1032 g, 4°C for 10 minutes. The whole serum sample was divided into three portions and then was frozen at -80°C for later analysis. Whole liver tissue was removed, cleaned and weighed. The right lobe of liver was cut to provide consistent liver samples in order to minimize interlobular variability in vitamin A or mineral distribution. One small piece of liver sample was excised from the right lobe portion and put in a glass vial containing 10% buffered formalin. Liver samples were immediately frozen at -80°C for later analysis. The right leg was cut to obtain a muscle sample and then frozen immediately at -80°C for later analysis. The birds from the second period were killed at the end of the experiment with serum and liver samples taken as previously described.

**Sample analysis** The methods for vitamin A, copper, zinc, iron, selenium, CP and calpain analysis were those outlined in chapter 2.

**Statistics** Statistical analyses were performed using the statistical software base SAS (SAS Institute Inc.). Data were assessed for homogeneity of variance using Analysis of Variance procedure with means compared by Student-Newman-Keuls (SNK) test at  $P < 0.05$ ; uneven number of replications were analyzed by using the General Linear Models procedure with SNK test from SAS.

## Results

The feeding of diets containing 5% tansy ragwort (TR) to male chicks caused a significant depression in body weight gain which was seen as early as one week (Figure 3.1). The average daily gain and final body weight were significantly different ( $P < 0.01$ ) between TR containing groups and control groups (Table 3.3). Adding copper and vitamin A to the diet had no influence on the growth rate. The liver weights were significantly lighter ( $P < 0.05$ ) (Figure 3.10) in all TR-containing groups than for the controls. In contrast, kidney weights were significantly heavier (Figure 3.11) in all TR-containing groups (Table 3.4). Copper and vitamin A supplementation of the diet had no effect on organ weights or gross pathology. In TR-containing groups, there was 16% mortality of birds in the TR alone and TR with vitamin A supplement groups. The livers were markedly changed from normal shape and became hard and a pale color, with about 15-100 ml ascites fluid. The gall bladders were extremely swollen. Tissue histopathology showed that the hepatic lobules were disorganized. Vacuolated hepatocytes, fibrosis, bile duct hyperplasia, megalocytes and veno-occlusion were seen (Appendice, Plate B,C,D), which are typical symptoms of TR poisoning (Cheeke and Shull, 1985).

The levels of several mineral elements in serum and liver were examined. The levels of copper in serum (Table 3.5) and liver (Table 3.6) were increased in TR-containing groups. However, the effect was significant only in the group with 250 ppm copper and 5% tansy ragwort in the diet, in which serum and liver copper concentrations had a 1.5 and 12 fold increase, respectively (Figure 3.4, 3.5). These results were similar to that

reported by Swick et al. (1982b) in rats. The levels of zinc in serum were all decreased in TR-containing groups with the exception of the TR alone group (Table 3.5). The liver zinc concentrations were significantly decreased in TR-containing groups compared to TR-free groups (Figure 3.7), but there were no significant differences when individual groups were compared with each other (Table 3.6). Contrasting results occurred with the liver iron levels. The liver iron content was significantly increased in TR-containing groups (Figure 3.8). The addition of TR and high levels of copper to the diet did not affect liver selenium levels (Table 3.6)(Figure 3.9). The same result has been seen in rats (Shull et al., 1979) and turkeys (Burguera et al., 1983).

The serum vitamin A levels were significantly depressed ( $P < 0.01$ ) in all TR-containing groups (Table 3.7). The extent of decrease ranged from 60-72% in four TR-containing groups (Table 3.2). Similar results in rats fed TR were reported earlier (Moghaddam and Cheeke, 1989). The liver vitamin A concentrations were significantly decreased ( $P < 0.05$ ) in TR-containing groups without vitamin A supplement, while there was a trend to decreased vitamin A concentration in the livers in TR-containing groups with the vitamin A supplement (Figure 3.3), but the differences were not significant (Table 3.7). Moghaddam and Cheeke (1989) reported that liver vitamin A levels were significantly decreased in rats fed TR. Adding copper to the diet did not have a significant influence on either liver or serum vitamin A concentration.

Calpain activity was significantly ( $P < 0.05$ ) depressed in TR-fed chicks (Table 3.8) (Figure 3.12). This may be due to PA inhibition of protein synthesis (Mattocks, 1986). Ceruloplasmin activities were not significantly different (Table 3.8) among groups with



the exception of the no TR and high level of vitamin A supplemented groups (Figure 3.13).

After removal of TR from the diet and provision of a high dietary levels of vitamin A supplement, the growth rate of chicks was rapidly increased (Figure 3.1). The vitamin A levels in the serum and liver were also significantly increased (Figure 3.14, 3.1.15). This suggests that although PA cause a irreversible liver damage, the ability to mobilize vitamin A from the liver to the blood is not irreversibly affected. Although the serum levels did not return completely to normal values in two weeks, one interpretation of these results is that PA inhibit liver synthesis of RBP, but when PA are removed from the diet, the ability to synthesize RBP is regained. Similarly, a marked increase in the liver vitamin A content occurred after TR feeding ceased (Figure 3.15), indicating a restoration of the ability to synthesize retinol-binding proteins.

## Discussion

Chickens fed TR developed typical signs of PA toxicosis (Appendice, Plate B, C, D). TR consumption resulted in depressed weight gain and feed efficiency (Table 3.3). These responses are typical of TR poisoning (Cheeke and Shull, 1985), and are due to functional impairment of intestine and liver cells (Hooper, 1975; Mattocks, 1986), adversely affecting nutrient absorption and metabolism, including inhibitory effects of PA on protein synthesis (Mattocks, 1986). The decreased liver and increased kidney weights reflect these metabolic effects. The same results have been found in the other species such as rats (Miranda et al., 1980) and mice (Pierson, 1978).

The tendency for increased liver concentrations of copper and iron and decreased zinc in chickens fed TR (Table 3.5, 3.6) is in agreement with previous observations with sheep (Bull et al., 1956; Swick et al., 1983), rats (Swick et al., 1982b, c), horses (Garrett et al., 1984) and rabbits (Swick et al., 1982a). Liver copper levels were increased by about 12 fold when both TR and high dietary copper were fed compared to either treatment alone. Similar results were reported by Swick et al. (1982b) who noted that accumulation of liver copper in rats fed *S. jacobaea* occurred only in the presence of high (250 ppm) copper levels. Australian workers (Howell et al., 1988) also reported that sheep fed heliotrope had elevated liver copper levels only when copper was also administered. All these results suggest that there is an interaction between dietary copper and PA intake, and possibly with the pattern of PA exposure. The biological mechanism for these interactions is still unknown. Impairment of normal subcellular copper excretion

may be one of the explanation for accumulation of copper in the liver. Swick et al.(1982b,c) reported that the copper concentration was markedly increased in the nuclei and debris fraction in TR- containing groups compared to the controls. The mobilization of copper from the liver was impaired with PA exposure. There is much evidence that newly absorbed copper is transported from the intestine loosely bound to albumin or certain amino acids. In this form the element is readily available to the liver and other tissues (Weiner and Cousins, 1980), in contrast to the much more tightly regulated distribution of ceruloplasmin-bound copper. This difference may explain why the liver copper concentrations were greatly increased with PA exposure and high dietary copper, while the ceruloplasmin activity was still maintained at normal levels (Table 3.8). Decreased excretion of copper into bile could also account for hepatic copper accumulation during PA intoxication (Swick et al., 1982b).

Tissue levels of copper and vitamin A showed no interaction in either the TR-containing or TR-free groups, in contrast to previous results obtained in rats (Moore et al., 1972; Sklan et al., 1987) in which there was a negative interaction. There may be a species difference in the metabolism of copper, vitamin A and PA.

The serum zinc concentration was significantly depressed in TR-containing groups compared to the controls. High levels of vitamin A or copper in the diet with PA had a further depressing effect on serum zinc concentration (Table 3.5, 3.6). Vitamin A has a stimulatory action on absorption of zinc in the intestinal ileum (Berzin et al., 1986). These workers reported that zinc transport in the chick ileum under the influence of vitamin A is based on the special binding with the zinc binding protein. Secondly, copper

has a higher affinity for metallothionein than zinc and may displace it (Mertz, 1986). In contrast, the iron concentration in the liver was significantly increased in TR-containing groups compared to TR-free groups, but there were no significant differences when individual groups were compared with each other (Table 3.6). Similar results were seen in rats (Swick et al., 1982b) and monkeys exposed to PA (Van der Watt et al., 1972). It has been noted that hematopoiesis is markedly impaired in the liver and bone marrow by PA toxicosis (Swick et al., 1982b,c; McLean, 1970). A likely explanation for these effects on iron metabolism is that PA may inhibit heme biosynthesis as a result of the alkylation of DNA by PA; the iron then cannot be used for hemoglobin formation, so the excess accumulates in the liver, spleen and other iron-storage tissues.

The vitamin A levels were significantly decreased in both serum and liver tissue (Table 3.7). Similar results were seen in rats fed TR (Moghaddam and Cheeke, 1989). The total liver vitamin A as a percentage of total supplemental vitamin A intake was calculated (Table 3.7). The data showed that less than half of total intake of vitamin A appeared in the liver in TR-containing groups compared to TR-free groups. This could suggest that the absorption of vitamin A is impaired in PA toxicosis. Several intestinal lesions including inhibition of crypt cell mitosis and villus atrophy occur in PA toxicosis (Hooper, 1975); This damage may impair nutrient absorption. The retinol-binding proteins are synthesized in the liver parenchymal cells, while PA damage occurred mainly in the same region. It is likely that RBP synthesis was inhibited by PA toxicosis, and the transport of vitamin A from parenchymal cells to the blood was depressed, resulting in low serum vitamin A values. Another possibility may be due to interaction

of vitamin A with metabolites of PA. Metabolites of PA such as pyrrole and t-4HH are very reactive oxidizing agents. PA exposure may alter the cellular antioxidants such as vitamin E, thus indirectly influencing tissue vitamin A. It is interesting that in TR-containing groups without vitamin A supplement, both serum and liver vitamin A concentrations were significantly decreased, but in TR-containing groups with vitamin A supplement, only the serum vitamin A was significantly ( $P < 0.01$ ) reduced (Table 3.7). The latter result may suggest that mobilization of vitamin A from the liver was inhibited by PA toxicosis. Hakansson and Hanberg (1989) reported that the dioxin TCDD can inhibit the storage of newly administered vitamin A from hepatocytes to stellate cells. The same mechanism with opposite results may occur in PA toxicosis. Batres and Olson (1987) reported that in rats with low liver vitamin A reserves, 83% of total liver vitamin A was found in parenchymal cells, while 82% of total liver vitamin A was found in stellate cells of rats with high liver vitamin A reserves. It is likely that in those TR-containing groups without vitamin A supplement, newly absorbed vitamin A was stored mainly in the parenchymal cells, which were impaired by PA toxicosis. This caused lower vitamin A concentrations in serum and liver (Table 3.2, 3.3). In the TR-fed groups with vitamin A supplement, the vitamin A probably was stored mainly in the stellate cells, so that mobilization of vitamin A was impaired by PA toxicosis, producing a low serum vitamin A and higher liver vitamin A levels. Furthermore, when PA was removed from the diet and then vitamin A was supplemented, the concentrations of vitamin A in serum and liver were greatly increased (Figure 3.14, 3.15) compared to TR-fed groups receiving vitamin A and TR at the same time (Table 3.2, 3.3), indicating that PA had

a reversible inhibition on the mobilization of vitamin A in the liver.

The ceruloplasmin activity was examined (Table 3.8). The inconsistency of data may be due to error of detection rather than the effects of dietary copper, vitamin A and TR. Ceruloplasmin in the vitamin A supplemented group without TR was greatly increased. It may be because vitamin A has an important role in the regulation of plasma ceruloplasmin values (Barber and Cousins, 1987).

The activity of calpain, an important  $\text{Ca}^{++}$  dependent enzyme involved in protein degradation, was significantly decreased ( $P < 0.05$ ) by feeding TR. It may be due to the inhibition of PA on protein synthesis. Another explanation may be that PA can inhibit the sequestration of  $\text{Ca}^{++}$  in extramitochondrial and mitochondrial compartments to produce an irreversible rise in the cytosolic free  $\text{Ca}^{++}$  concentration (Griffin and Segall, 1989), which may change the activity of calpain.

Table 3.1. Experimental design for male chickens

Treat. No.	Basal diet(%)	Ground corn(% diet)	Tansy ragwort(% diet)	Copper (ppm)	Vitamin A (IU/kg diet)	Birds/ treat.
1	95	5	0	0	0	12
2	95	0	5	0	0	12
3	95	5	0	250	0	12
4	95	0	5	250	0	12
5	95	5	0	0	25,000	12
6	95	0	5	0	25,000	12
7	95	5	0	250	25,000	12
8	95	0	5	250	25,000	12

Table 3.2 Composition of the basal diets (OSU #1704 and 1705)

ingredients	1704(%)	1705(%)
Yellow corn	58.35	63.52
Fat	2.00	2.00
Soybean meal	32.25	27.50
Meat and bone meal	5.00	5.00
Alfalfa meal	1.00	1.00
Defluo. phosphate	0.42	0.25
Limestone flour	0.35	0.13
Salt	0.25	0.25
Trace mineral (mix-65)*	0.05	0.05
Vitamin premix (I-75)*	0.20	0.20
D,L-methionine	0.13	0.10
Total	100.00	100.00

\* The premixes supplied the following quantities per kg feed:

Vitamin premix: vitamin A 3,300 IU; vitamin D 1,100 IU; vitamin E 1.1 IU; vitamin K 0.55 mg; vitamin B-12 0.0055mg; riboflavin 3.3 mg; pantothenic acid 5.5 mg; niacin 22 mg; choline chloride 220 mg; folic acid 0.22 mg; ethoxyquin 64.43 mg.

Trace mineral: Calcium 107.5 mg; manganese 60 mg; iron 20 mg; zinc 28 mg; copper 2 mg; iodine 1.2 mg; cobalt 0.205.



Table 3.3 Growth and feed efficiency of male chicks fed copper, vitamin A and tansy ragwort

Dietary treatment			Ave. initial wt.(g)	Ave. final wt.(g)	Ave. daily gain (g)	Feed efficiency (F/G)*
% dietary tansy ragwort	Cu (ppm)	Vitamin A (IU/kg)				
0	0	0	147.6±2.8	1667.3±51.2 <sup>ab</sup>	54.3±1.7 <sup>ab</sup>	1.787
5	0	0	148.6±5.6	632.0±23.2 <sup>c</sup>	17.4±0.8 <sup>c</sup>	2.483
0	250	0	147.2±4.0	1604.2±36.5 <sup>b</sup>	52.0±1.3 <sup>b</sup>	1.802
5	250	0	148.5±4.1	667.5±33.1 <sup>c</sup>	18.5±1.2 <sup>c</sup>	2.363
0	0	25,000	147.9±5.7	1643.3±38.9 <sup>ab</sup>	53.4±1.3 <sup>ab</sup>	1.822
5	0	25,000	147.3±4.5	675.0±25.6 <sup>c</sup>	18.8±0.9 <sup>c</sup>	2.552
0	250	25,000	148.4±6.1	1750.9±47.5 <sup>a</sup>	57.3±1.6 <sup>a</sup>	1.781
5	250	25,000	148.3±5.1	700.0±29.0 <sup>c</sup>	19.7±1.0 <sup>c</sup>	2.629

Means ± SE in the same column followed by different superscripts are different P < 0.05)

\* feed intake (kg)/body weight (kg)

Table 3.4 Liver and kidney weights as percentage of final body weight of male chicks fed copper, vitamin A and tansy ragwort

Dietary treatment			Liver weight (%)	Kidney weight (%)
% dietary tansy ragwort	Cu (ppm)	Vitamin A (IU/kg)		
0	0	0	2.270±.07 <sup>a</sup>	0.765±.02 <sup>a</sup>
5	0	0	1.649±.10 <sup>b</sup>	1.226±.08 <sup>b</sup>
0	250	0	2.326±.08 <sup>a</sup>	0.762±.03 <sup>a</sup>
5	250	0	1.829±.21 <sup>b</sup>	1.248±.12 <sup>b</sup>
0	0	25,000	2.266±.11 <sup>a</sup>	0.820±.03 <sup>a</sup>
5	0	25,000	1.805±.07 <sup>b</sup>	1.191±.07 <sup>b</sup>
0	250	25,000	2.346±.14 <sup>a</sup>	0.754±.03 <sup>a</sup>
5	250	25,000	1.791±.08 <sup>b</sup>	1.170±.10 <sup>b</sup>

Means ± SE in the same column followed by different superscripts are different (P<0.05)

Table 3.5 Copper and zinc concentration in serum of male chicks fed copper, vitamin A and tansy ragwort

Dietary treatment			Serum copper (ppm)	Serum Zinc (ppm)
% dietary tansy ragwort	Cu (ppm)	Vitamin A (IU/kg)		
0	0	0	0.158±.01 <sup>b</sup>	1.762±.06 <sup>ab</sup>
5	0	0	0.201±.02 <sup>ab</sup>	1.387±.13 <sup>b</sup>
0	250	0	0.153±.01 <sup>b</sup>	1.614±.12 <sup>ab</sup>
5	250	0	0.236±.02 <sup>a</sup>	0.987±.07 <sup>c</sup>
0	0	25,000	0.173±.01 <sup>b</sup>	1.963±.08 <sup>a</sup>
5	0	25,000	0.176±.01 <sup>b</sup>	0.974±.13 <sup>c</sup>
0	250	25,000	0.153±.01 <sup>b</sup>	1.618±.11 <sup>ab</sup>
5	250	25,000	0.206±.02 <sup>ab</sup>	0.863±.12 <sup>c</sup>

Means ± SE in the same column followed by different superscripts are different (P < 0.05)

Table 3.6 Copper, zinc, iron and selenium concentrations in liver (DM) of male chicks fed copper, vitamin A and tansy ragwort

Dietary treatment			Liver copper (ppm)	Liver zinc (ppm)	Liver iron (ppm)	Liver Se. (ppb)
% dietary tansy ragwort	Cu (ppm)	Vitamin A (IU/kg)				
0	0	0	11.09±1.3 <sup>a</sup>	61.48±6.06	338.3±53.7	3245.4 ±109.6
5	0	0	17.44±2.9 <sup>a</sup>	54.56±8.88	529.6±108.4	3481.4 ±51.6
0	250	0	14.01±1.3 <sup>a</sup>	65.16±7.34	434.1±65.9	3914.3 ±811.8
5	250	0	167.58±45.2 <sup>b</sup>	52.31±3.99	514.2±103.1	4235.8 ±385.6
0	0	25,000	10.83±.94 <sup>a</sup>	64.78±6.16	405.3±94.6	
5	0	25,000	13.96±1.2 <sup>a</sup>	49.32±3.96	568.0±106.1	
0	250	25,000	12.34±.63 <sup>a</sup>	54.18±5.01	319.0±37.9	
5	250	25,000	161.98±29.2 <sup>b</sup>	40.74±7.23	413.9±59.4	

Means ± SE in the same column followed by different superscripts are different (P < 0.05)

Table 3.7 Vitamin A concentration in serum and livers of male chicks fed copper, vitamin A and tansy ragwort

Dietary treatment			serum vitamin A (IU/ml)	Liver vitamin A* (IU/g)(DM)	Total liver vitamin A (IU)(wet tissue)	Total liver vitamin A as % of total supple- mental Vit.A intake
% dietary tansy ragwort	Cu (ppm)	Vitamin A (IU/kg)				
0	0	0	2.688±.23 <sup>b</sup>	436.56±64.70 <sup>b</sup>	4401.59 ±171.8 <sup>a</sup>	25.2
5	0	0	1.059±.20 <sup>c</sup>	287.37±72.07 <sup>c</sup>	754.7±52.0 <sup>b</sup>	9.6
0	250	0	3.261±.11 <sup>ab</sup>	474.16±30.90 <sup>b</sup>	4971.41 ±211.4 <sup>a</sup>	29.1
5	250	0	0.902±.14 <sup>c</sup>	188.77±33.06 <sup>c</sup>	502.7±48.6 <sup>b</sup>	6.3
0	0	25,000	3.988±.21 <sup>a</sup>	4807.8±212.0 <sup>a</sup>	49080.0 ±2818.4 <sup>c</sup>	72.07
5	0	25,000	1.128±.28 <sup>c</sup>	4305.4±542.1 <sup>a</sup>	13420.0 ±651.2 <sup>d</sup>	39.89
0	250	25,000	3.808±.21 <sup>a</sup>	5764.0±548.1 <sup>a</sup>	62900.0 ±3930.5 <sup>c</sup>	89.7
5	250	25,000	1.038±.31 <sup>c</sup>	4891.8±814.6 <sup>a</sup>	15740.0 ±595.38 <sup>d</sup>	43.39

Means ± SE in the same column followed by different superscripts are different (P < 0.05)

\* Comparison was made in Log values.

Table 3.8 Ceruloplasmin and calpain activity in serum and muscle of male chicks fed copper, vitamin A and tansy ragwort

Dietary treatment			Ceruloplasmin (U/ ml)*	Calpain (U/g)
% dietary tansy ragwort	Cu (ppm)	Vitamin A (IU/kg)		
0	0	0	6.083±1.18 <sup>bc</sup>	2.718±0.18 <sup>a</sup>
5	0	0	5.979±1.01 <sup>bc</sup>	2.079±0.21 <sup>b</sup>
0	250	0	9.649±1.50 <sup>b</sup>	
5	250	0	4.500±0.44 <sup>bcd</sup>	
0	0	25,000	18.438±3.88 <sup>a</sup>	
5	0	25,000	4.729±0.98 <sup>bcd</sup>	
0	250	25,000	3.625±0.55 <sup>cd</sup>	
5	250	25,000	2.583±0.50 <sup>d</sup>	

Means ± SE in the same column followed by different superscripts are different (P < 0.05)

\* Comparison was made in Log values.

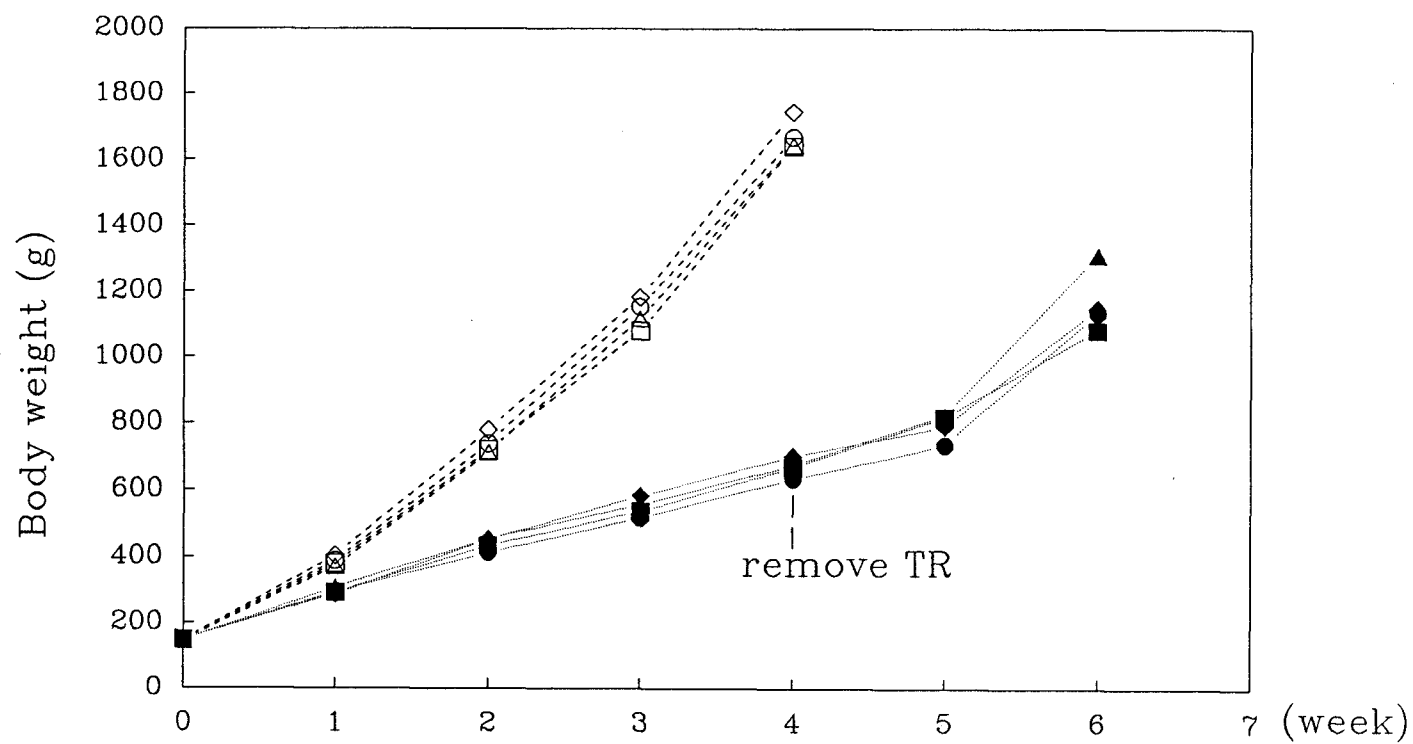


Figure 3.1 Growth rate in male chicks fed tansy ragwort (TR)

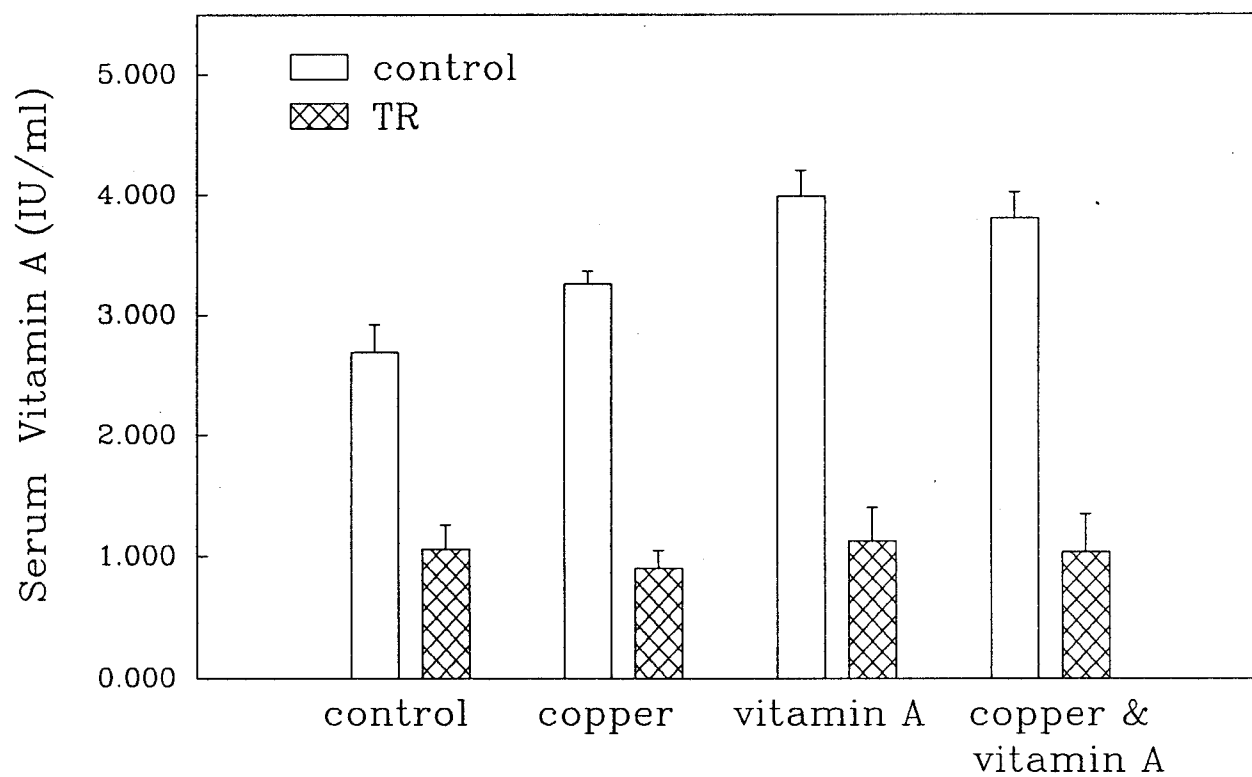


Figure 3.2 Serum vitamin A levels in male chicks fed tansy ragwort (TR)



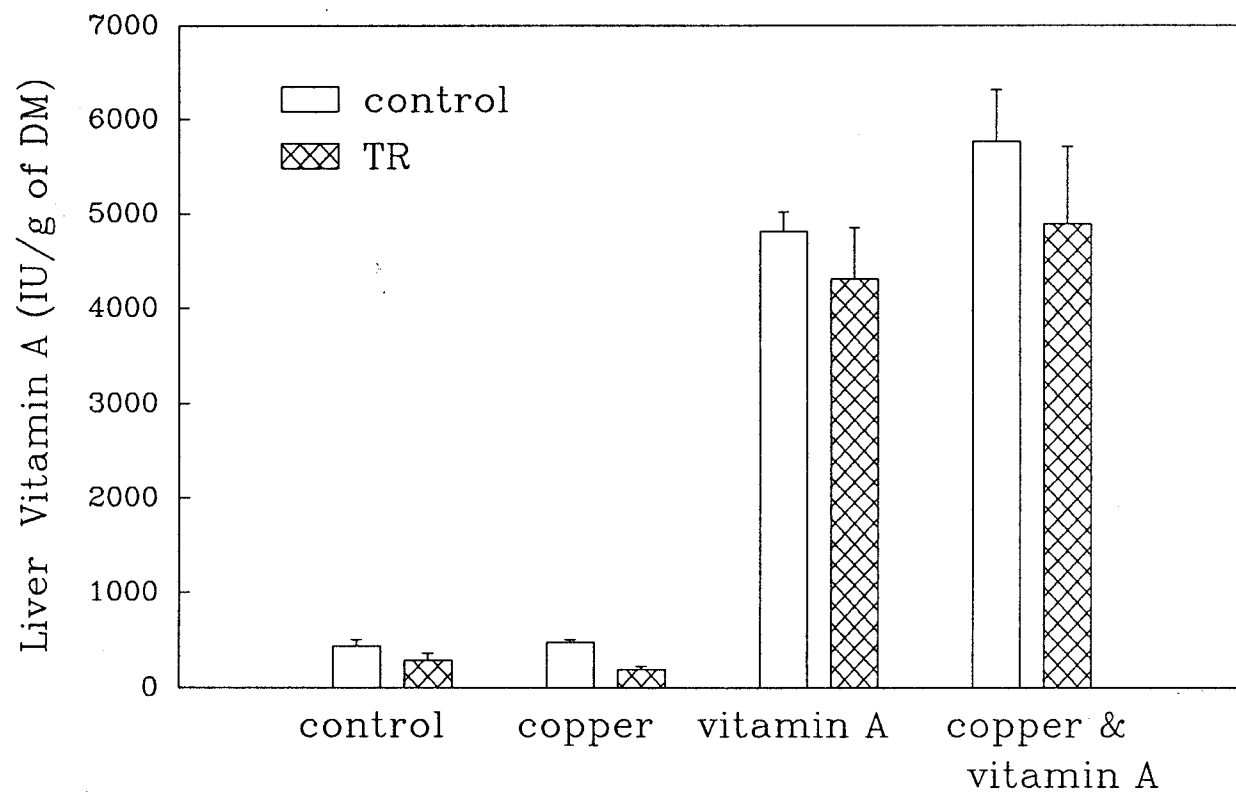


Figure 3.3 Liver vitamin A levels in male chicks fed tansy ragwort (TR)

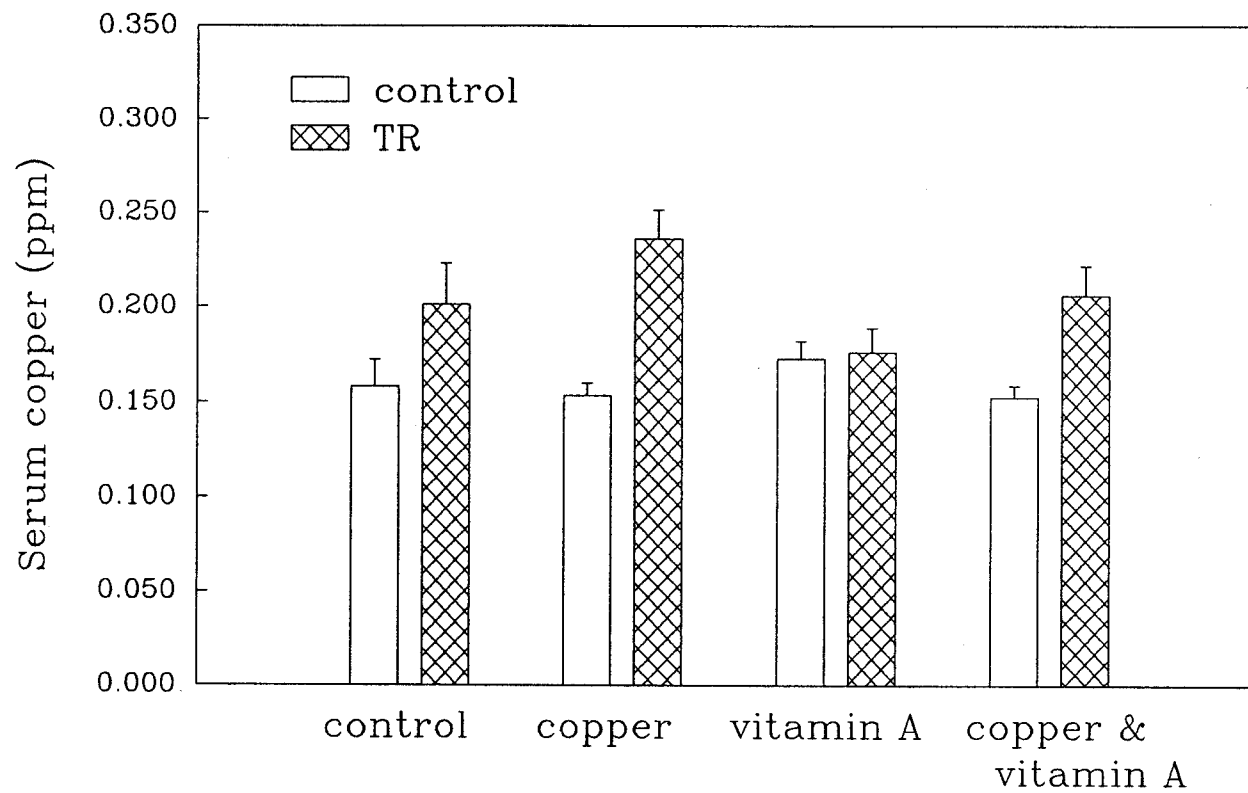


Figure 3.4 Serum copper levels in male chicks fed tansy ragwort (TR)

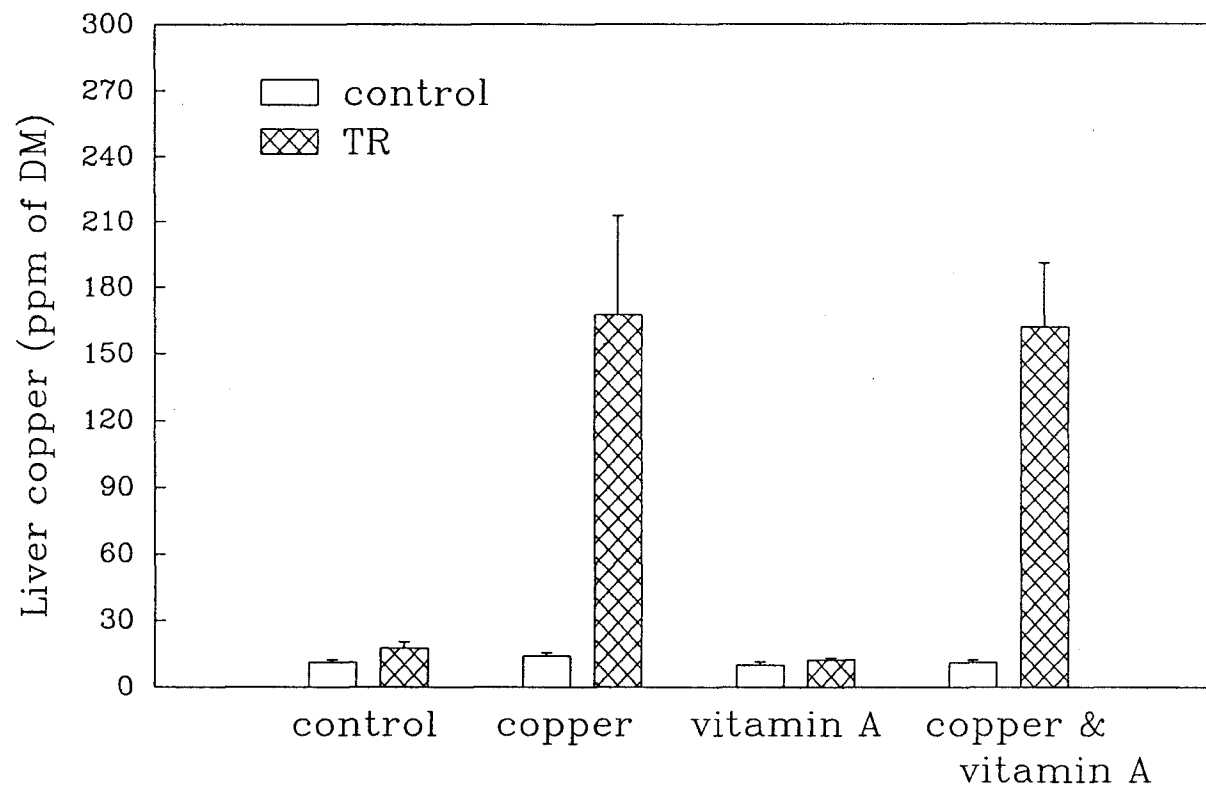


Figure 3.5 Liver copper levels in male chicks fed tansy ragwort (TR)

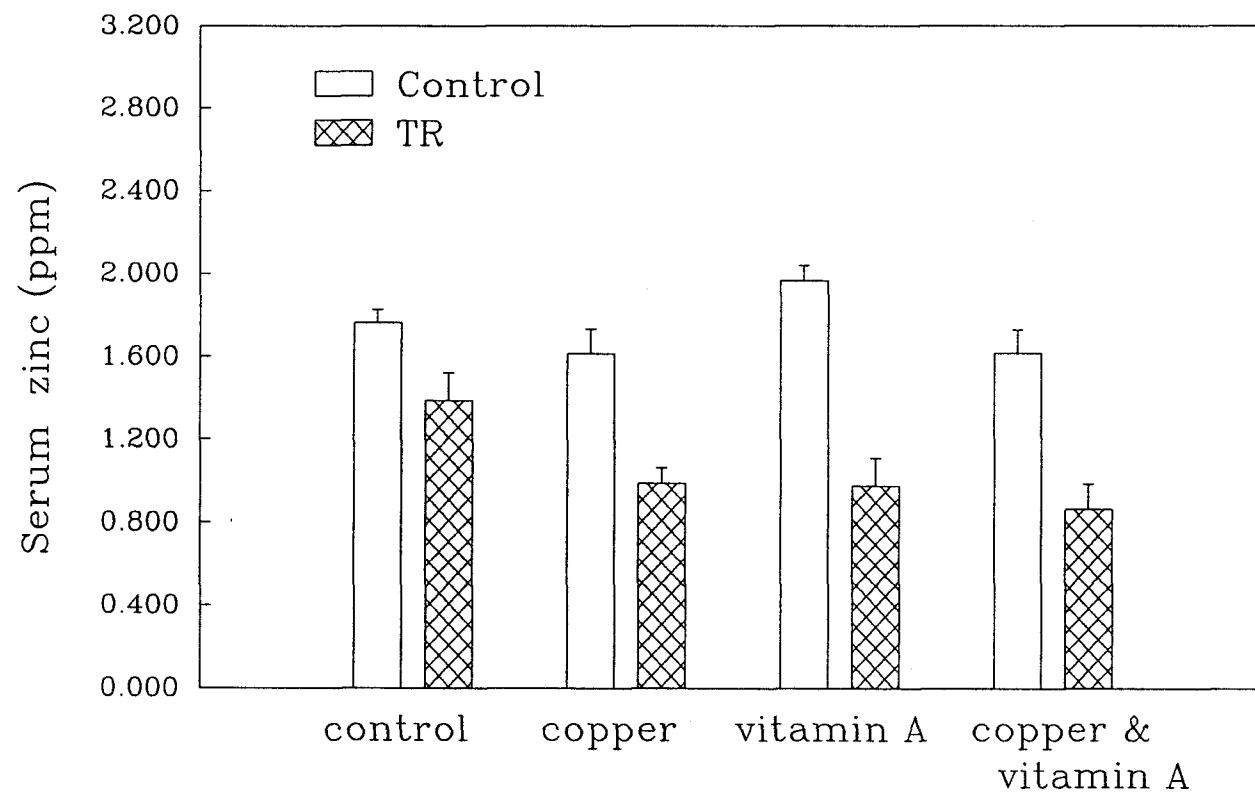


Figure 3.6 Serum zinc levels in male chicks fed tansy ragwort (TR)

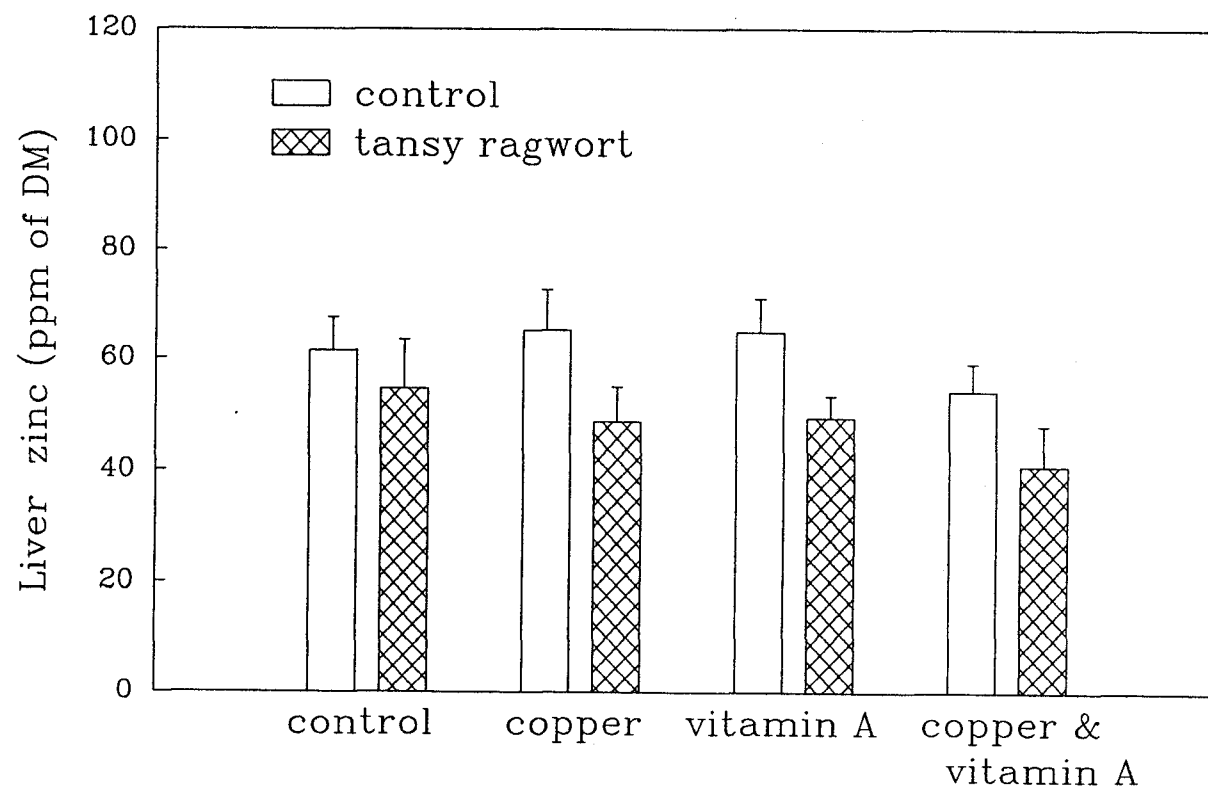


Figure 3.7 Liver zinc levels in male chicks fed tansy ragwort

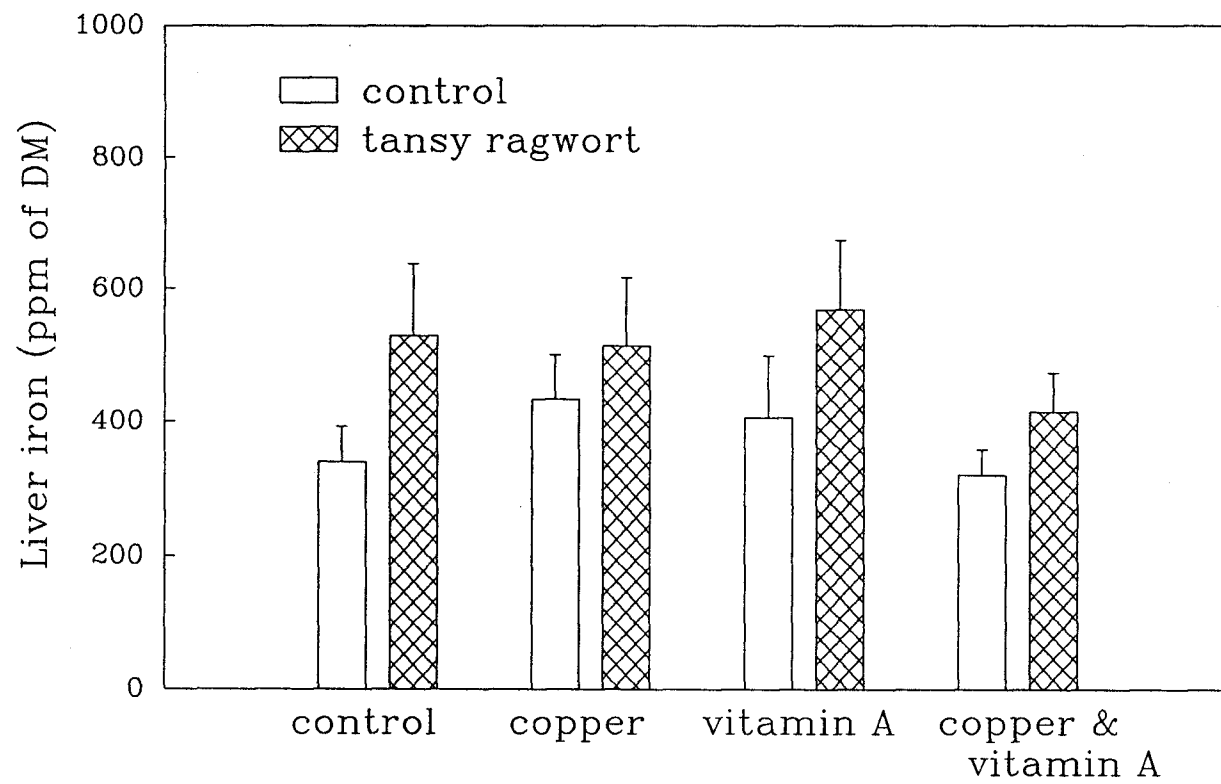


Figure 3.8 Liver iron levels in male chicks fed tansy ragwort

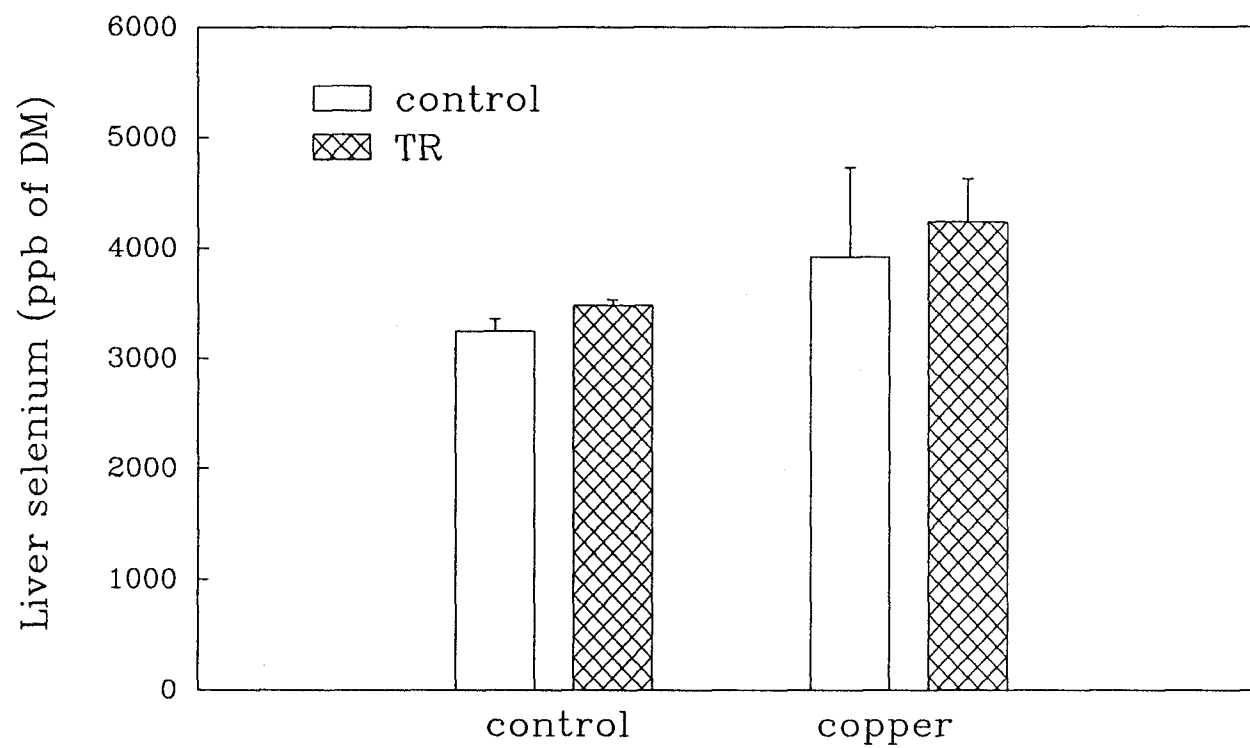


Figure 3.9 Liver selenium levels in male chicks fed tansy ragwort (TR)

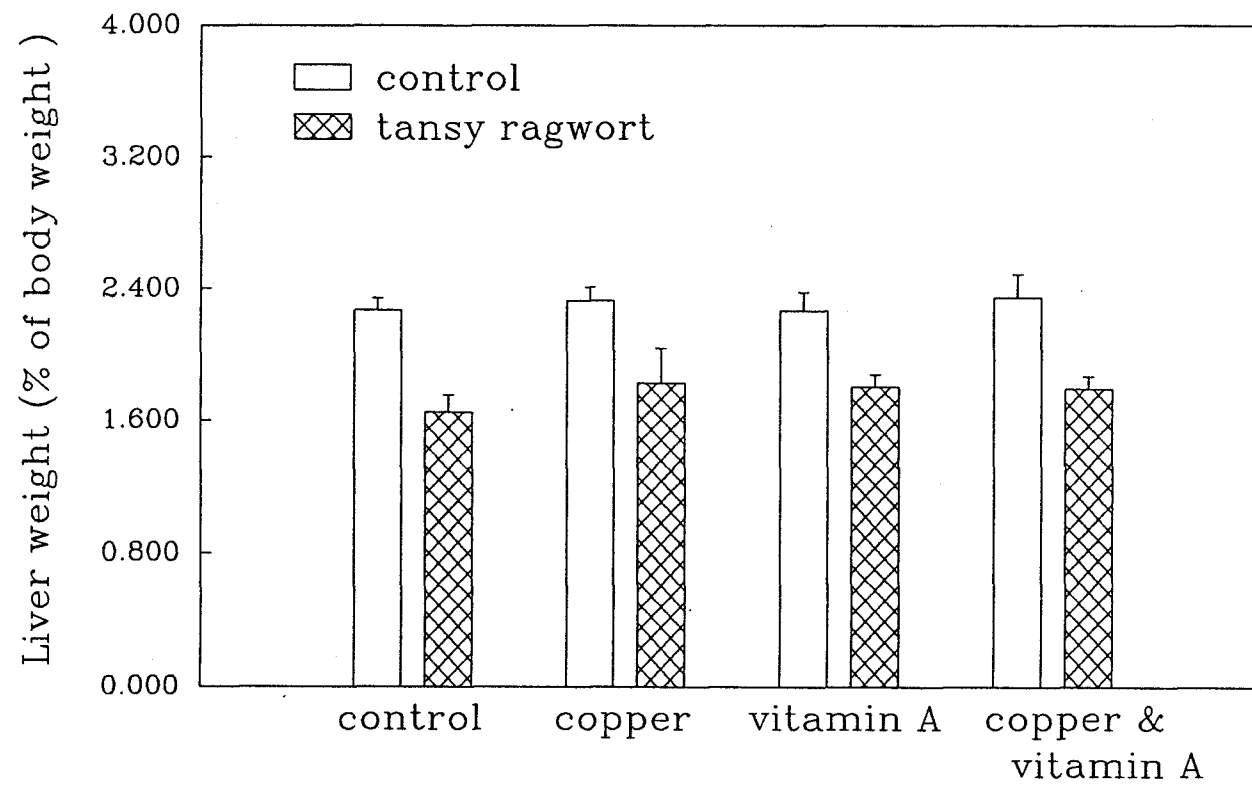


Figure 3.10 Liver weights as percentage of body weight of male chicks fed tansy ragwort



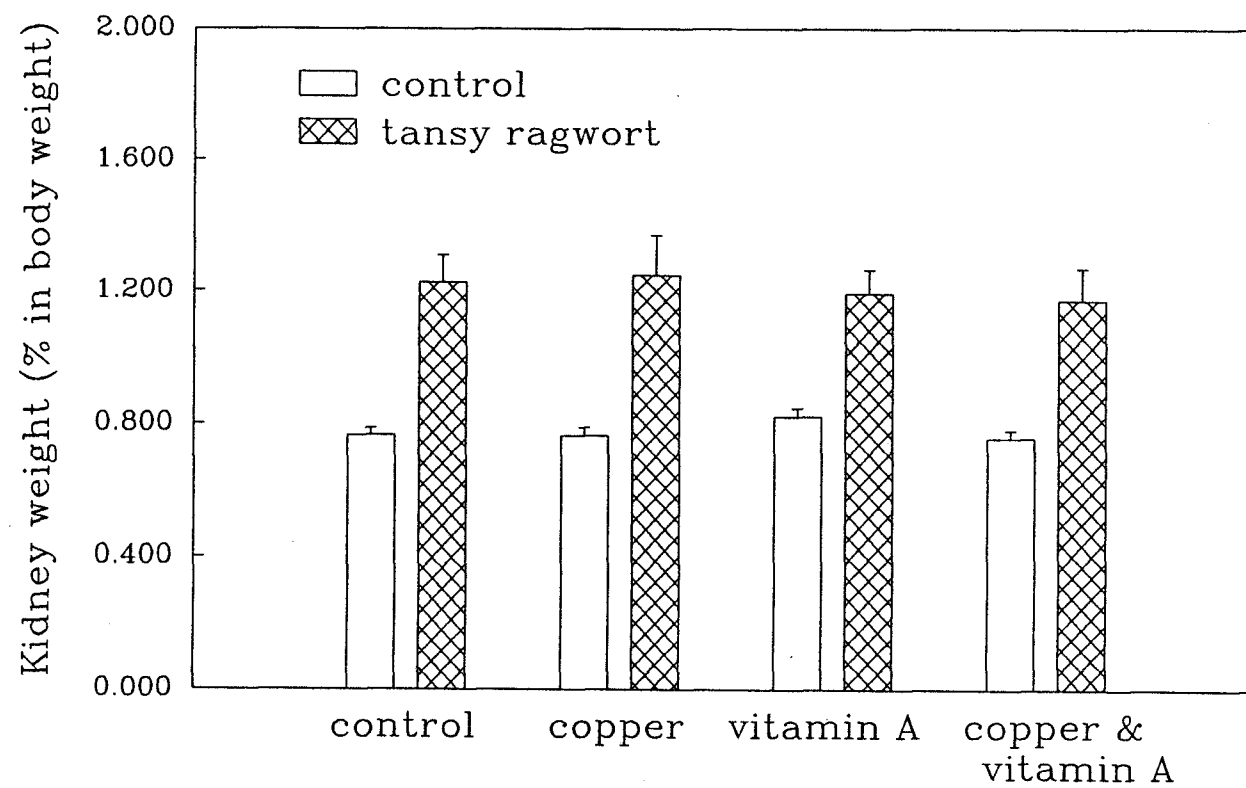


Figure 3.11 Kidney weights as percentage of body weight of male chicks fed tansy ragwort

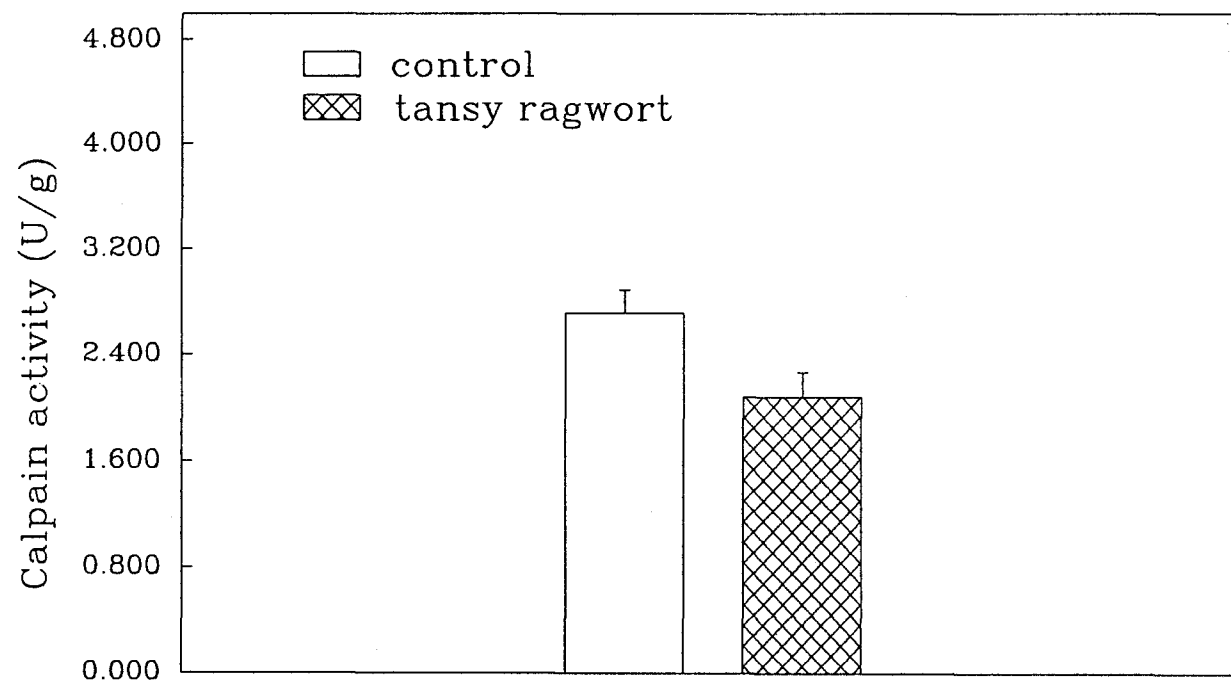


Figure 3.12 Calpain activity in leg muscle of male chicks fed tansy ragwort

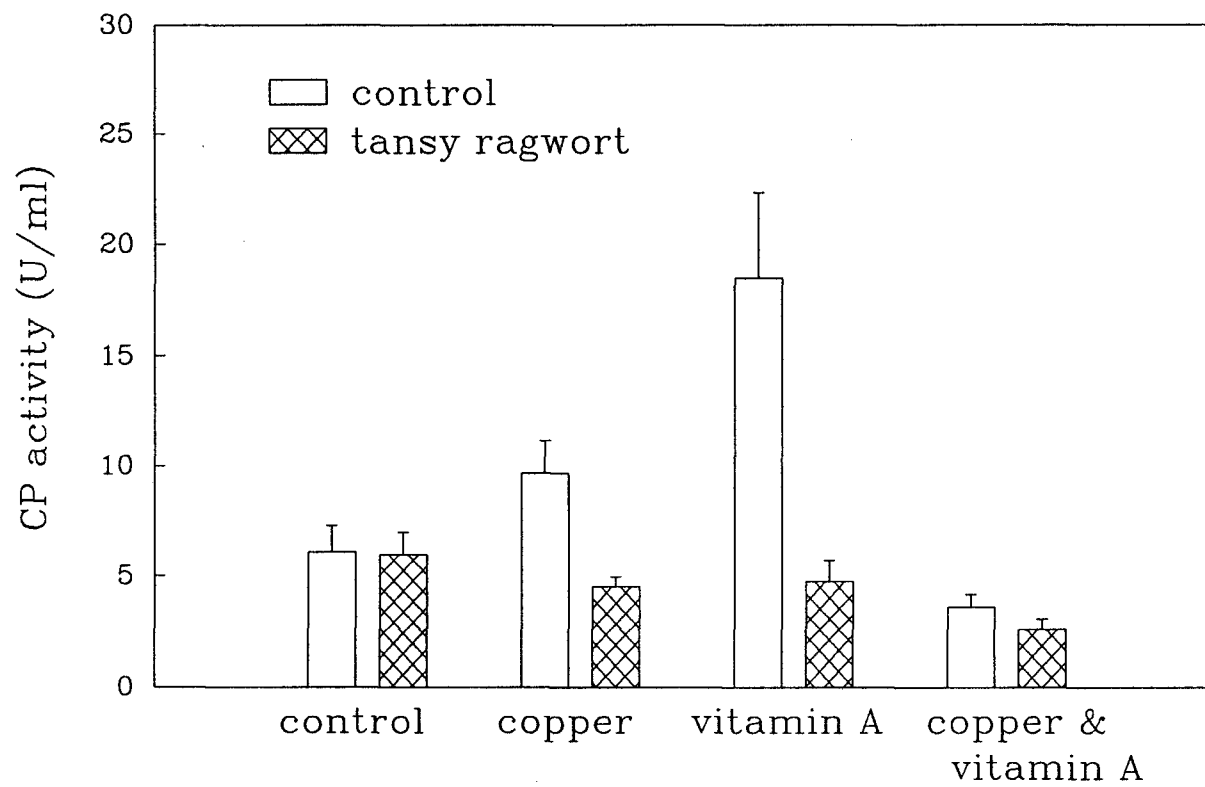


Figure 3.13 Ceruloplasmin activity in male chicks fed tansy ragwort

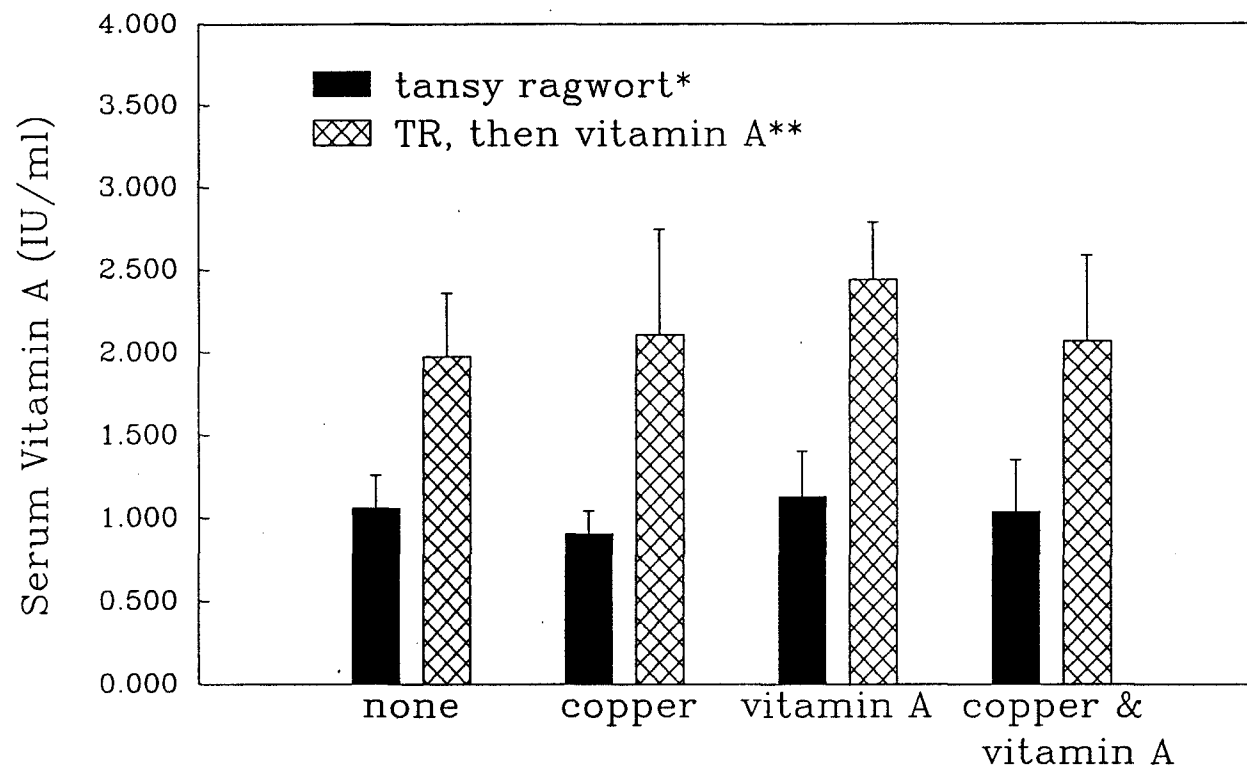


Figure 3.14 Serum vitamin A levels in male chicks fed tansy ragwort (TR)

\* Tansy ragwort: Fed tansy ragwort diet for four weeks.

\*\* TR, then vitamin A: Fed tansy ragwort for four weeks followed by a high vitamin A diet for two weeks.

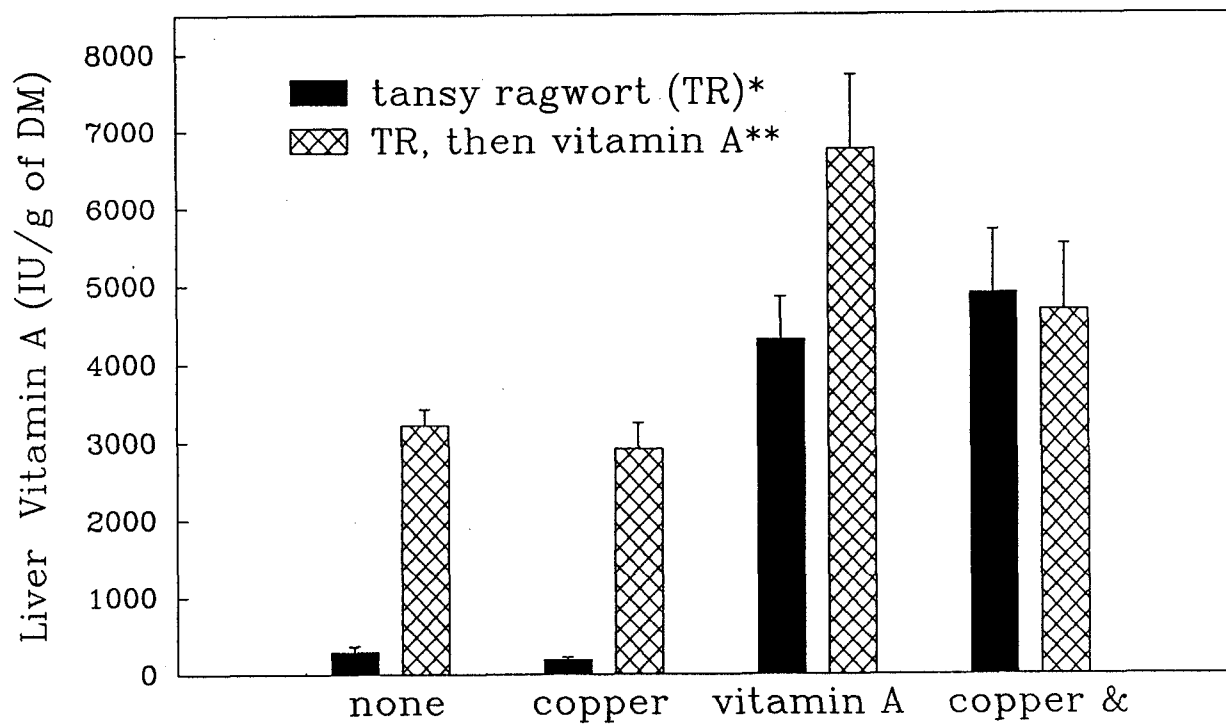


Figure 3.15 Liver vitamin A levels in male chicks fed tansy ragwort

\* Tansy ragwort: Fed tansy ragwort diet for four weeks.

\*\* TR, then vitamin A: Fed tansy ragwort for four weeks followed by a high vitamin A diet for two weeks.

### 3.2 Effects of dietary pyrrolizidine alkaloids on retention of previously stored vitamin A in the chicken

#### Summary

The effect of feeding the pyrrolizidine alkaloid (PA)-containing plant tansy ragwort (TR)(*Senecio jacobaea*) on retention of previously stored vitamin A in the chicken was examined. In the first period, two groups of chicks were fed a diet containing 25,000 IU vitamin A/kg diet for two weeks, to load their tissues with vitamin A. Following this period, the basal diet (OSU# 1705) with or without 5% TR was fed, to determine if PA influenced the mobilization of stored vitamin A from the liver to blood. Blood samples were taken at 4 day intervals for 24 days. By day 8, serum vitamin A levels were significantly depressed ( $P < 0.05$ ). After 24 days of PA exposure, serum vitamin A levels were reduced by 55% and 8.5% in the TR-fed group and the control group, respectively. Liver concentration of vitamin A (IU/g) was increased ( $P < 0.05$ ) at day 24 of TR feeding, because of the effects of reduced of vitamin A mobilization. In the control group, liver vitamin A concentration was decreased by 13% over the same period. The results indicate that PA inhibit the mobilization of previously stored vitamin A from the liver, probably by inhibiting hepatic synthesis of retinol-binding proteins.

## Introduction

Many pyrrolizidine alkaloids (PA) present in *Senecio*, *Crotalaria*, *Heliotropium* and *Echium* species are well known hepatotoxins (Cheeke and Shull, 1985; Mattocks, 1986). The main symptoms of hepatotoxic effects of PAs are swollen hepatocytes, progressive fibrosis, bile duct proliferation, veno-occlusion and loss of hepatic metabolic function (Cheeke and Shull, 1985). Consumption of PA containing plants has long been known to affect mineral (primarily copper) metabolism in several species (Bull et al., 1956; Swick et al., 1982a, 1982b; Garrett et al., 1984). The mechanisms are still not completely understood. Effects of PA on plasma and liver vitamin A levels have also been reported (Moghaddam and Cheeke, 1989). The postulated mechanisms have been suggested to be inhibition of retinol-binding protein synthesis and impairment of vitamin A absorption. Because retinol and retinyl esters are stored mainly in stellate cells (Blomhoff et al., 1985), whereas the PAs effects are primarily in hepatocytes (Mattocks, 1986), it is not clear whether PA have a direct effect on liver storage of vitamin A. Furthermore, Segall et al (1985) suggested that the ultimate metabolites of PA are reactive aldehydes which act as oxidizing agents. If so, PA exposure may alter the cellular antioxidants such as vitamin E resulting in an indirect influence on tissue vitamin A status. Feeding synthetic antioxidants has a pronounced protective effect against PA toxicosis (Miranda et al., 1981a, 1981b). On the other hand, the metabolites of PA such as pyrrole and t-4HH have been identified to be strong oxidants, which may cause peroxidation of membranes (Segall et al., 1985; Griffin and Segall, 1989). The effects

of vitamin A on membrane structure and function are well known and various parameters like membrane integrity, permeability of lysosomal and mitochondrial membranes, nucleotide uptake and cell surface charge are affected by vitamin A (Sridhararao, 1987). A PA-vitamin A interaction could have important implications in human and animal nutrition.

The objective of this experiment was to investigate the effect of PA on liver retention of previously stored vitamin A, to attempt to more closely identify the mode of action of PA in reducing serum vitamin A levels.



## Materials and Methods

**Animals** The eggs were obtained from the Keith Smith farm, Hot Springs, AR. After hatching, feather sexing and a two week pre-experiment period, fifty two-week old female broiler chickens (Peterson x Arbor Acres) were randomly assigned to two groups and housed in electrically heated ventilated wood pens with feed and water *ad libitum*. They were maintained at 18-22°C under 24 hours artificial light. In the first period (two weeks) both groups were fed the same experiment diet. After the second week one group was fed basal diet and the other group was fed the diet with 5% tansy ragwort for another 24 days. Birds were wing banded and weighed individually initially and at the end of each week.

**Feed** During the first period, OSU ration 1705 (Table 3.2) was used as the basal diet in both groups. All *trans* retinol palmitate (Sigma Chemical Company), with a concentration of 500,000 IU/g, was mixed with the basal diet to supply 25,000 IU added vitamin A supplement per kilogram of diet. After the second week, vitamin A supplement was removed from both groups, and one group continued on OSU ration 1705, while the other group was fed the diet with 5% tansy ragwort (Table 3.9.). The feed intake was calculated at the end of the experiment.

**Sample preparation** At the end of the second week, three birds from each group were killed with injection of T-61 euthanasia solution (Hoechst-roussel Agri-Vet company). Serum and liver samples were taken as previously described above and stored at -80°C for further analysis. After the second week, six birds were marked and blood samples

taken from the wing vein each four days for a total 24 days in both groups. The serum was immediately separated at 1032 g, 4°C for 10 minutes (Sorvall RC-3 with HG-4L head) and then frozen at -80°C. At the end of the experiment, these birds were killed with injection of euthanasia solution. Blood samples were collected, and then serum was separated and stored in -80°C. Livers were removed and weighed. One small piece of liver sample was cut from the right lobe and put in 10% buffered formalin for histopathology. The livers were immediately frozen at -80°C for later analysis.

**Sample analysis** The method for vitamin A analysis described in chapter 2 was used.

**Statistics** Statistical analyses were performed using the statistical software base SAS (SAS Institute Inc.). Some data were analysed by using repeated measures analysis of variance with  $P < 0.05$  a criterion of significance in General Linear Models procedure. Other data were assessed by using *two-T* test from statgraph.

## Results

There were no significant differences in the growth rate of chicks in the first period when the basal diet containing 25,000 IU/kg vitamin A was fed, but it was markedly different between the two groups after 24 days of PA exposure (Figure 3.16). The significant difference appeared as early as the eighth day after starting PA exposure ( $P < 0.05$ )(Figure 3.17)(Table 3.10). Similar results were also seen in serum vitamin A. In the period of TR feeding, serum vitamin A levels were significantly decreased in the TR group compared to the control ( $P < 0.01$ )(Table 3.11). The decline in the serum vitamin A concentration appeared at the same time as the decreased growth rate (Figure 3.18). Similar results have been reported in rats by Moghaddam and Cheeke (1989). After 24 days of PA exposure, the serum vitamin A levels were 55% lower in the TR-containing group compared to the levels before TR feeding began ( $P < 0.01$ ), while the control group only had a 8.5% decline ( $P > 0.05$ )(Figure 3.19). In contrast, the liver vitamin A levels had a 35% increase in the TR-containing group compared to initial values ( $P < 0.05$ ), whereas the control group had a 13% decrease in the liver vitamin A concentration (Figure 3.20). This indicates that the mobilization of previously stored vitamin A from the liver was inhibited by PA.

The liver and kidney weights expressed as a percentage of final body weight were not different between the two groups ( $P > 0.1$ )(Figure 3.21). The tissue histopathology showed that in the chicks fed high vitamin A before TR exposure, the liver lesions were much less severe than for the chicks fed TR without pre-feeding vitamin A, with much

less vacuolated hepatocytes, fibrosis and megalocytosis than in birds not fed high vitamin A (Experiment 1, section 3.1). The liver lobules were quite uniform, and some mitotic figures were seen in the hepatocytes (Appendix, Plate E), in contrast to previous results obtained in Experiment 1 (section 3.1). It is well known that PA can inhibit mitotic division in hepatocytes (Cheeke and Shull, 1985); therefore pre-feeding high levels of vitamin A may have protective effects against PA toxicosis in animals.

## Discussion

Consumption of TR by chicks pre-fed high levels of vitamin A caused a depression of body weight gain (Figure 3.16). The depression of body weight gain was noted by the eighth day after starting PA exposure (Table 3.10)(Figure 3.17). Acute liver damage and inhibition of protein synthesis in the liver are possible reasons for the growth depression. However, the liver and kidney weights as percentages of final body weight did not change in the TR-containing group compared to the control, in contrast to previous results obtained in section 3.1. Histopathology results (Appendix IV) also suggested possible protective effects of prior exposure to high vitamin A.

The depression of serum vitamin A concentration occurred at the same time as the decrease in growth rate (Figure 3.18). Similar results were also found in rats fed PA (Moghaddam and Cheeke, 1989). This may indicate that PA effects on vitamin A metabolism can occur in the early stages of PA toxicosis. This could be significant in human exposure to PA containing products. The consumption of "health-food" products containing PA such as comfrey can cause pathology such as veno-occlusive disease (Ridker et al., 1985). The results obtained from this experiment suggest that serum vitamin A levels might be a sensitive indicator of PA exposure, and might have potential for diagnostic purposes.

After 24 days of PA exposure, the serum vitamin A levels had declined by 55% and 8.5% in TR-fed and the control birds, respectively. However, in the TR-fed group, liver vitamin A levels was 35% higher than at the beginning, while liver vitamin A

concentration was 13% lower than at the beginning in the control birds. This suggest that PA exposure interferes with the mobilization of liver vitamin A into the blood. When adequate vitamin A is available in the diet, most of liver storage of vitamin A is stored in the stellate cells (Batres andf Olson, 1987). In order to transport of vitamin A to the target tissues, vitamin A in the liver has to be mobilized from stellate cells to hepatocytes, where it binds to RBP and then is released into the blood. Another view suggested by Blomhoff et al. (1990) is that stellate cells can synthesize RBP and mobilize retinol-RBP directly into the plasma without prior transfor of retinol to the hepatocytes. The hepatotoxic dioxin TCDD can inhibit the transfer of newly administered vitamin A from hepatocytes to stellate cells (Hakansson and Hanberg, 1989). If so, the results obtained from this experiment and previous results in section 3.1 may show that PA acts on the mobilization of vitamin A either by inhibiting the transfer of stored vitamin A from stellate cells to parenchymal cells or by inhibiting the synthesis of RBP resulting in no carrier for liver vitamin A transference.

In this experiment, the total liver vitamin A concentration was not significantly different between the treatment and the control groups (Table 3.11), while in the previous results in section 3.1, the total liver vitamin A in TR-fed birds with vitamin A supplement was only 27% of that of controls. The results also suggest that the absorption of vitamin A may be inhibited by PA toxicosis, as also observed in rats fed TR (Moghaddam and Cheeke, 1989).

Table 3.9 Experimental design for female chickens

## First period (14 days)

Treat. No.	Basal diet(%)	Ground corn(%)	Tansy ragwort(%)	Vitamin A (IU/kg)	birds/ treat.
1	100	0	0	25,000	25
2	100	0	0	25,000	25

## Second period (24 days)

1	95	5	0	0	22
2	95	0	5	0	22

Table 3.10 Growth rate of female chicks administered a high dose (25,000 IU/kg diet) of vitamin A prior to 24 days of exposure to 5% dietary tansy ragwort

Item	Body weight (g)	
	Control	5% tansy ragwort
Initial B.W.	382.3±4.5 <sup>a</sup>	381.8±4.2 <sup>a</sup>
After 2 wks. administered high levels of vitamin A	1045.0±4.3 <sup>a</sup>	1055.0±11.5 <sup>a</sup>
Start feeding tansy ragwort		
0 day	1045.0±4.3 <sup>a</sup>	1055.0±11.5 <sup>a</sup>
4 days	1280.0±14.1 <sup>a</sup>	1226.7±20.4 <sup>a</sup>
8 days	1433.3±14.1 <sup>a</sup>	1281.7±32.8 <sup>b</sup>
12 days	1723.3±19.6 <sup>a</sup>	1368.3±41.3 <sup>b</sup>
16 days	1925.0±22.8 <sup>a</sup>	1383.3±60.2 <sup>b</sup>
20 days	2130.0±24.1 <sup>a</sup>	1355.0±73.4 <sup>b</sup>
24 days	2311.7±31.0 <sup>a</sup>	1346.7±88.5 <sup>b</sup>

Means ± SE in the same row followed by different superscripts are different (P < 0.05)



Table 3.11 Levels of vitamin A of female chicks administered a high dose (25,000 IU/kg diet) of vitamin A prior to 24 days of exposure to 5% dietary tansy ragwort

Item	Vitamin A levels	
	Control	5% tansy ragwort
<b>In Serum:</b>		
(IU/ml)		
0 day	4.051±0.15 <sup>a</sup>	4.196±0.18 <sup>a</sup>
4 days	4.103±0.32 <sup>a</sup>	4.147±0.14 <sup>a</sup>
8 days	3.947±0.13 <sup>a</sup>	3.501±0.12 <sup>b</sup>
12 days	3.690±0.25 <sup>a</sup>	3.140±0.21 <sup>a</sup>
16 days	3.862±0.10 <sup>a</sup>	2.758±0.16 <sup>b</sup>
20 days	3.872±0.21 <sup>a</sup>	2.363±0.07 <sup>b</sup>
24 days	3.706±0.11 <sup>a</sup>	1.863±0.13 <sup>b</sup>
<b>In Liver:</b>		
(IU/g)(DM)		
0 day	3935.52±201.62 <sup>**</sup>	3935.52±201.62 <sup>**</sup>
24 days	3412.70±124.31 <sup>a</sup>	5343.24±596.84 <sup>b</sup>
<b>In total liver:</b>		
(IU)(wet tissue)		
0 day	26015.3±992.6 <sup>**</sup>	26015.3±992.6 <sup>**</sup>
24 days	41630.12±1490.1 <sup>b</sup>	38573.77±2906.9 <sup>b</sup>

Means ± SE in the same row followed by different superscripts are different (P<0.05)

\* The 0 day value is the mean of six birds (three from each group).

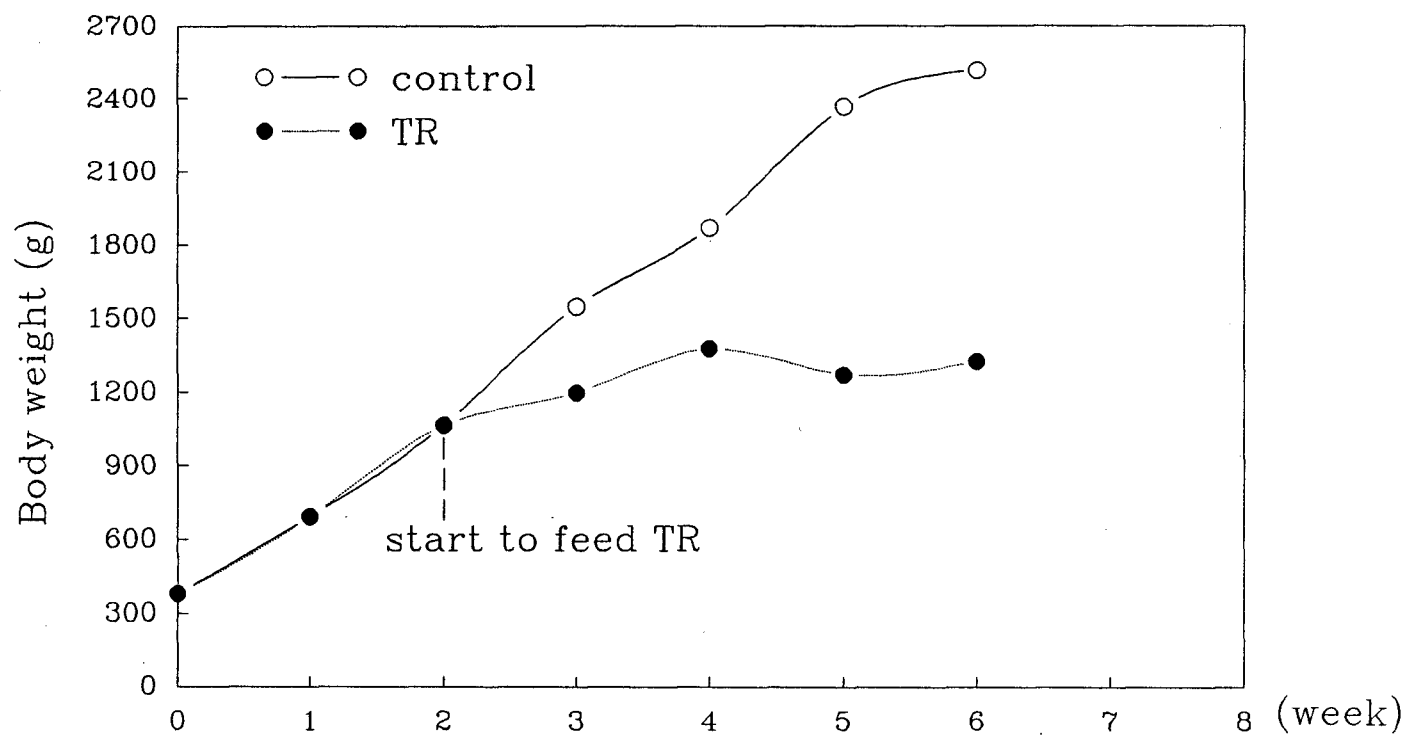


Figure 3.16 Growth rate of chicks fed a high vitamin A diet followed by a tansy ragwort (TR)-containing diet

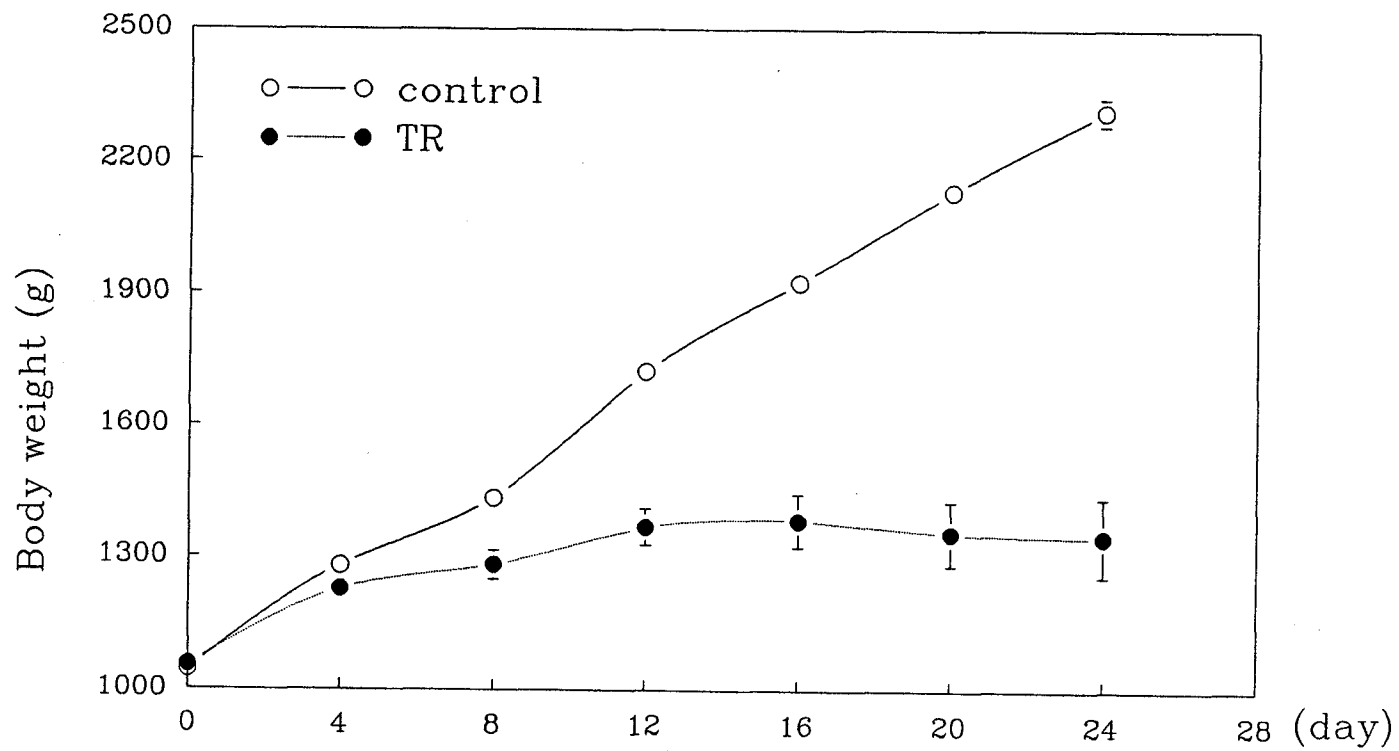


Figure 3.17 Growth rate of chicks pre-fed a high vitamin A diet followed by a control or TR-containing diet (TR feeding commenced on day 0)

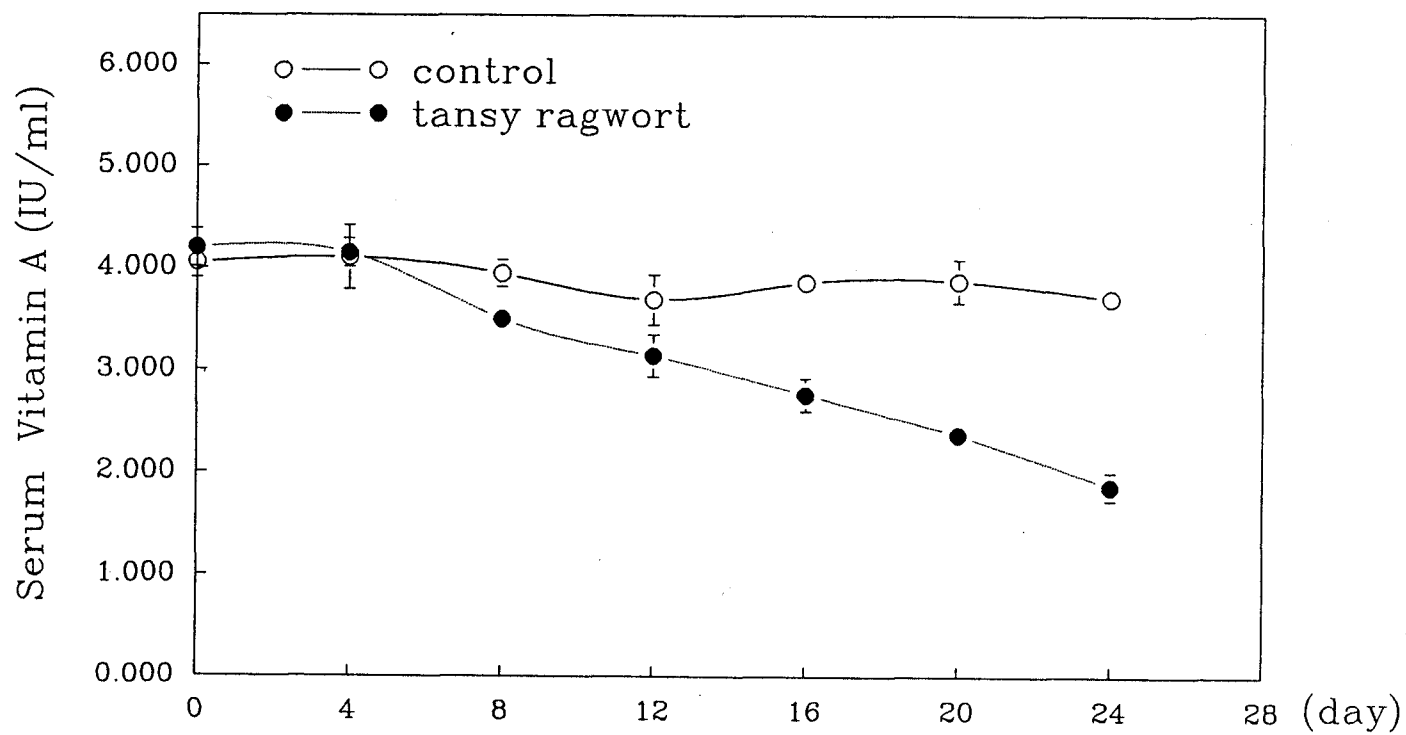


Figure 3.18 Serum vitamin A of chicks pre-fed a high vitamin A diet followed by a control or TR-containing diet (TR feeding commenced on day 0)

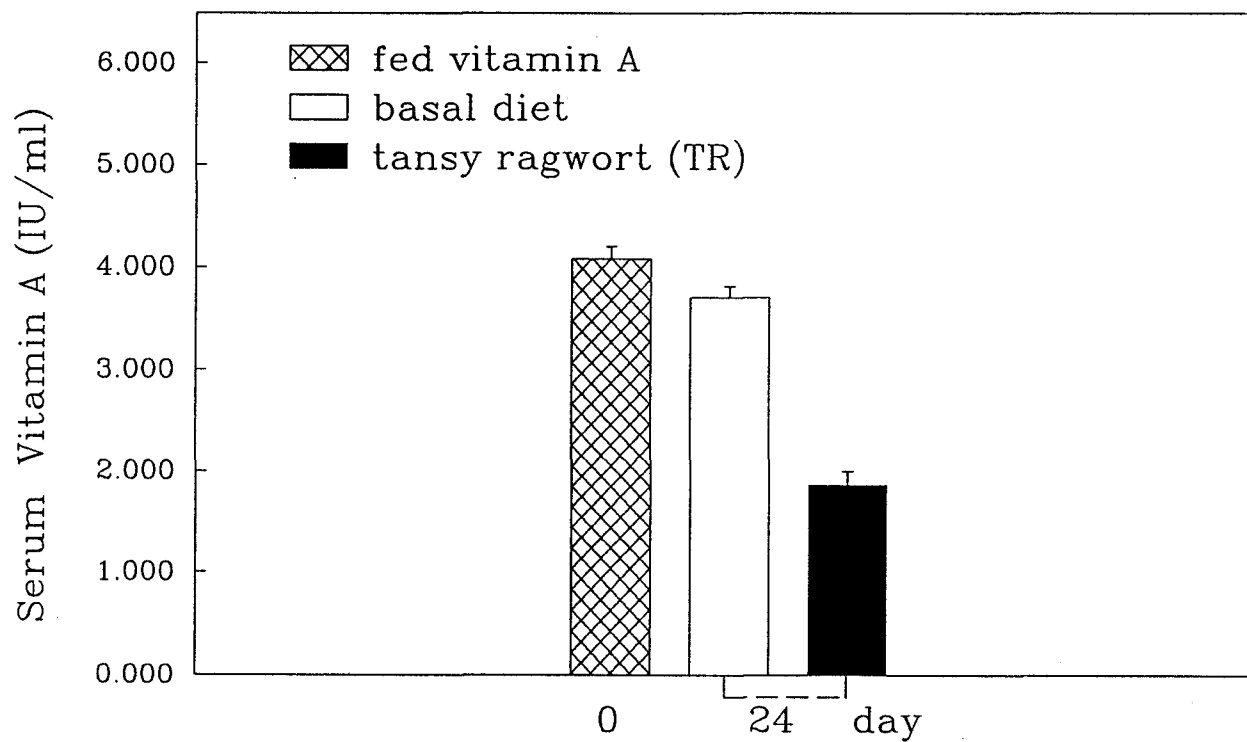


Figure 3.19 Serum vitamin A of chicks pre-fed a high vitamin A diet followed by a control or TR-containing diet on day 0 and 24

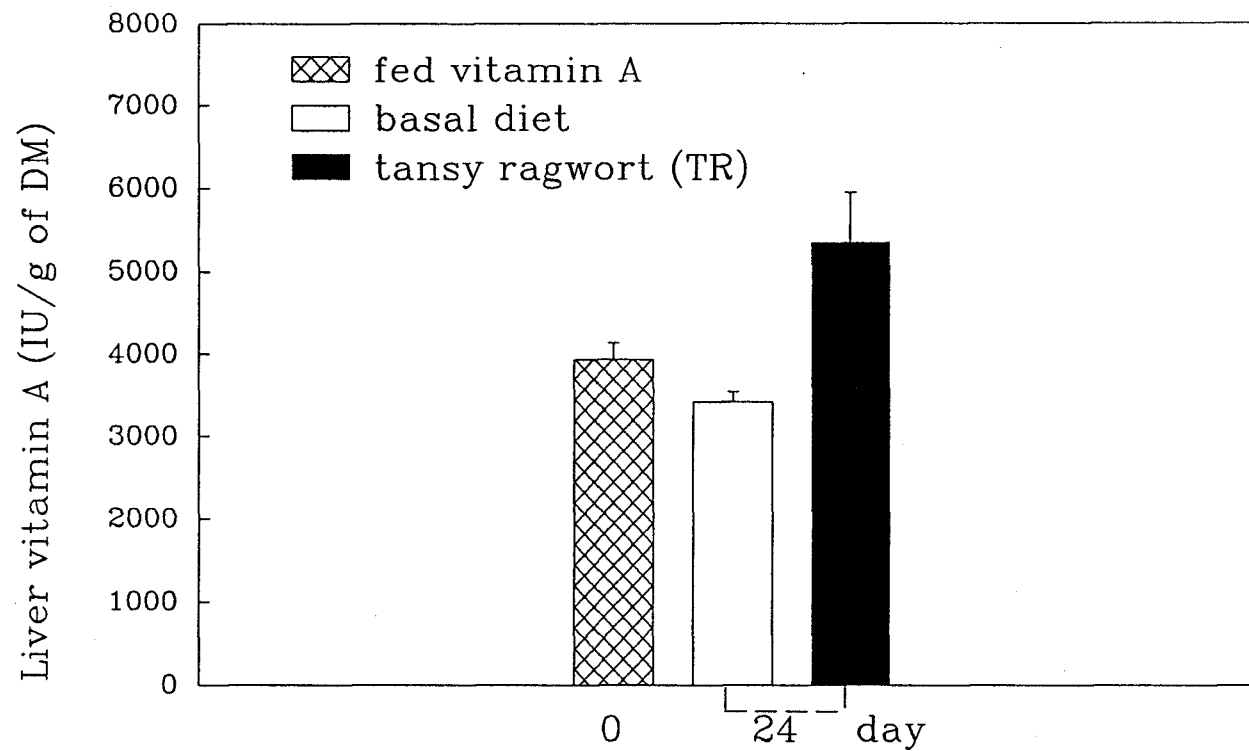


Figure 3.20 Liver vitamin A of chicks pre-fed a high vitamin A diet followed by a control or TR-containing diet on day 0 and 24

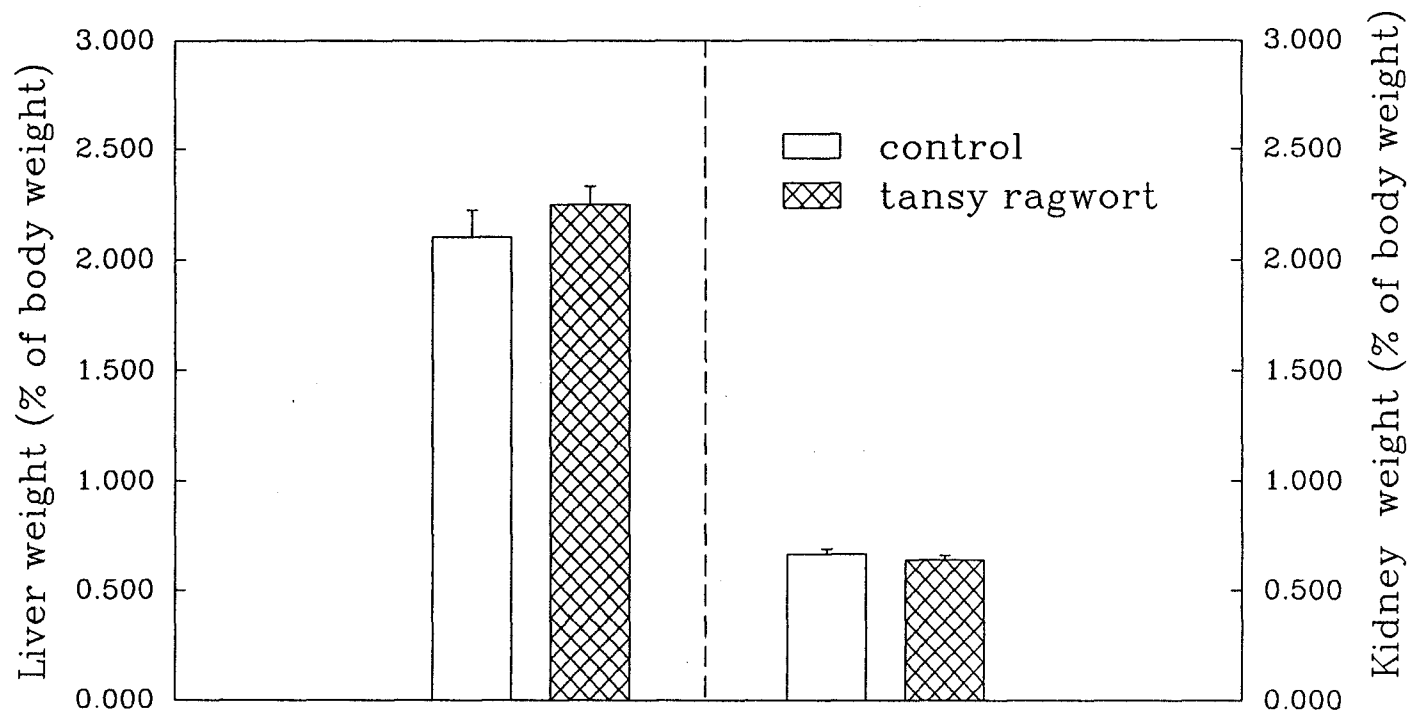


Figure 3.21 Liver and kidney weights as percentage of final body weight of chicks pre-fed high vitamin A diet followed by a control or TR-containing diet

### 3.3 Effects of dietary pyrrolizidine alkaloids on copper and vitamin A tissue levels in Japanese quail

#### Summary

The effect of feeding the pyrrolizidine alkaloid (PA)-containing plant tansy ragwort (TR)(*Senecio jacobaea*) on copper and vitamin A tissue levels in Japanese quail was examined. Japanese quail are highly resistant to the hepatotoxic effects of PA. The possible interaction between copper and vitamin A was also investigated. The experiment was a 2x2x2 factorial design with added 0 and 5% tansy ragwort, 0 and 250 ppm copper, and 0 and 25,000 IU/kg diet vitamin A. Consumption of TR did not affect the growth rate of Japanese quail. There were no significant differences in the serum copper concentrations among all treatment groups. Liver copper levels were decreased with TR feeding ( $P < 0.05$ ). The concentrations of zinc and iron in the serum and liver were normal in TR-fed groups compared to the controls. There was no significant effect ( $P > 0.05$ ) on the serum vitamin A concentration. The liver vitamin A concentrations were also not significantly different with the exception of the basal TR group. Copper supplementation of the diet increased serum vitamin A levels ( $P < 0.05$ ). The lack of major effects of TR on mineral and vitamin A tissue levels in this PA resistant species suggest that hepatotoxicity is necessary to induce the changes in tissue levels of copper and vitamin A seen in PA-susceptible species.



## Introduction

The pyrrolizidine alkaloids (PA) are a large and important family of natural toxicants produced by a variety of plant species. Most PA-containing plants which produce toxic effects in livestock and humans are in the genera *Senecio*, *Crotalaria*, *Heliotropium* and *Echium* (Cheeke and Shull, 1985).

The hepatotoxicity and metabolism routes of PA are well known (Cheeke and Shull, 1985; Mattocks, 1986). They also have an important interaction with nutrient metabolism. An interaction between PA toxicosis and minerals (primarily copper) has been noted. The evidence arose from the observation that consumption of *E. plantagineum* and *H. europaeum* by sheep led to excessive liver copper concentrations followed by the hemolytic crisis of copper toxicity (Bull et al., 1956). Since then, other workers have noted that PA exposure causes elevated liver copper and iron and decreased zinc in horses (Garrett et al., 1984), rabbits (Swick et al., 1982a) and rats (Swick et al., 1982b, 1982c). However, elevated liver copper levels in PA-poisoned animals do not always occur. Swick et al. (1983) and White et al. (1984) did not find elevated liver copper levels in sheep consuming *S. jacobaea*. Liver copper concentrations were normal in cattle with chronic heliotrope poisoning (Bull, 1961). Species differences in susceptibility to PA toxicosis are well known (Cheeke and Shull, 1985). Both PA-susceptible species such as horses (Garrett et al., 1984) and rats (Swick et al., 1982b, 1982c), and PA-resistant animals such as sheep (Bull et al., 1956; Seaman 1987) and rabbits (Swick et al., 1982a) have been observed to have increased hepatic copper levels when fed sources of PA.

However, Swick et al.(1982b) found that elevated liver copper levels in rats fed *S. jacobaea* occurred only in the presence of high levels of copper (50 and 250 ppm) and with high PA intakes. Similar results have been shown by Howell et al. (1988) who observed that heliotrope consumption caused elevated levels of liver copper in sheep only when copper was also administered. Is the occurrence of hepatotoxic effects of PAs necessary to influence copper metabolism? This question has yet to be conclusively answered.

In addition, it has been reported that the concentration of copper and vitamin A in liver have an inverse relationship. Factors that increase the concentration of one usually decrease the concentration of other (Moore et al., 1972). Rechman et al.(1987) reported that in copper deficient rats, retinol and retinyl esters increase significantly in the liver and decrease in the serum, while Sklan et al.(1987) claimed that high levels of vitamin A in the diet result in increasing copper concentration in the liver and decreasing copper concentration in the serum.

The objective of this experiment was to study the effects of PA on copper and vitamin A levels in serum and liver in Japanese quail, a species that is highly resistant to the hepatotoxic effects of PA (Buckmaster et al., 1977); to determine if there is an association between the PA-induced changes in tissue mineral and vitamin A levels and hepatic damage.

## Materials and Methods

**Animals** Eighty-nine Japanese quail of mixed sex, two week-old of age, were housed in electrically heated brooder batteries with experimental feed and water provided *ad libium*. They were randomly assigned to eight groups (Table 3.12.) and maintained on 24 hours artificial light at  $30 \pm 2^{\circ}\text{C}$ . Birds were wing banded and body weights were weighed individually in initially and at the end of each week. The experiment lasted six weeks.

**Feed** Eight different test diets were used. The basal diet (90% of total, OSU #1876) is shown in Table 3.13. The remaining 10% of the diet was supplemented with vitamin A or copper mixed with ground corn or tansy ragwort to bring up the diet to 100%. Tansy ragwort was collected in the bloom stage near Corvallis, Oregon, air dried, ground through a 1 mm screen in a wiley mill and incorporated into the diet in order to make up 0 and 10% of diet. All-*trans* retinol palmitate (Sigma Chemical Company) with a potency of 500,000 IU/g was used as the vitamin A supplement. Cupric sulfate (anhydrous powder, Spectrum Chemical, MFG. Corp.) was used as the copper supplement (Table 3.12.). Feed intake data was collected at the end of the experiment.

**Sample preparation** At the end of the experiment, six birds from each group were killed with injection of T-61 enthanasia solution (Hoechst-Roussel Agri-Vet Company). Blood samples were immediately taken by cardiac puncture from each bird, and serum was separated in a automatic-refrigerated centrifuge (Sorvall RC-3 with HG-4L head) at 1032 g,  $4^{\circ}\text{C}$  for 10 minutes. The serum samples were divided into three portions and

then frozen at  $-80^{\circ}\text{C}$  for future analysis. Whole liver tissue was removed, trimmed and weighed. The right lobe of the liver was cut to provide consistent liver samples in order to minimize interlobular variability in vitamin A and mineral distribution. Liver samples were immediately frozen at  $-80^{\circ}\text{C}$  for later analysis.

***Sample analysis*** The methods for vitamin A, copper, zinc, iron and CP analysis previously outlined in chapter 2 were used.

***Statistics*** Statistical analysis was performed using the statistical software base SAS (SAS institute Inc.). Data were assessed for homogeneity of variance using Analysis of Variance procedure with means compared by Student-Newman-Keuls (SNK) test at  $P < 0.05$ ; uneven number of replications were analyzed by using the General Linear Models procedure with SNK test from SAS.

## Results

The inclusion of 10% tansy ragwort (TR) in the diet fed to Japanese quail did not cause significant depression in body weight gain (Figure 3.22). The average weekly gain and final body weights were slightly lower with no significant differences ( $P > 0.05$ ) between TR-fed groups compared to the control groups (Table 3.14). Adding copper and vitamin A to the diet had no influence on the growth rate. The liver weights were not different in TR-containing groups compared to the controls. Similar results indicating high resistance to PA toxicosis have been obtained in a previous Japanese quail trial (Buckmaster et al., 1977). In the TR-containing groups, the gross examination did not show any differences between TR-fed and the control groups. There was no mortality attributable to diet in any treatments.

The concentrations of copper, zinc and iron in serum and liver have been summarized on Table 3.15, 3.16. The levels of copper in serum were not different among treatment groups ( $P > 0.05$ ). Copper concentration in the liver was significantly decreased ( $P < 0.05$ ) in TR-containing groups compared to the control groups, but there was no significance when individual groups were compared with each other. Adding high levels of vitamin A supplement with TR caused a significant depression on liver copper concentration compared to the control group. The levels of zinc in serum were not different in all treatment groups with the exception of a pair of comparison between vitamin A supplement alone and vitamin A supplement plus copper (Table 3.16). The data from Table 3.16 showed that TR did not affect liver zinc concentration, while

copper supplement in the diet caused a significant depression in the zinc concentration. There were no significant differences in the iron concentration of serum and liver among treatment groups (Table 3.16).

There were no significant differences in the serum vitamin A concentration ( $P > 0.05$ ) among treatment groups (Table 3.17). The liver vitamin A concentrations were also not significantly different in all treatment groups (Figure 3.24) with exception of the TR alone group (Table 3.17). Adding copper to the diet induced an increase of serum vitamin A ( $P < 0.05$ ) (Figure 3.23).

Ceruloplasma activity was not significantly different among all treatment groups (Table 3.15).

## Discussion

Consumption of TR did not affect the growth rate of Japanese quail. Performance data showing no adverse effects of 10% TR were in agreement with previous results (Buckmaster et al., 1977). The feed efficiency for the TR-fed groups was slightly lower than for controls (Table 3.14), probably due to the lower energy content in the TR-containing diet. Similar results were also seen in the previous work (Buckmaster et al., 1977).

There were no significant differences in the serum copper concentrations among treatment groups, but the liver copper levels were decreased overall with TR treatment. This contrasts with the marked increase in liver copper concentration in the chicks fed TR. In work with Merino sheep, there were only slight hepatotoxic effects with a long-term exposure to *E. plantagineum* and no elevation in the liver copper concentration was noted (Culvenor et al., 1984). Bull (1961) reported that liver copper concentrations were normal in cattle suffering chronic heliotrope poisoning. These results may suggest that the hepatotoxic effects of PAs are necessary to influence copper metabolism. Japanese quail are very resistant to the PA and may consume over 2000% of body weight of TR with no pathological signs (Buckmaster et al., 1977). In this experiment, the total TR intake per bird was only 55% of body weight. This small TR intake may be not enough to cause liver damage. The concentrations of zinc and iron in the serum and liver also were not affected during PA exposure in this experiment. An antagonism between copper and zinc in their metabolism may be one of reasons (Mertz, 1986). The higher affinity of copper

for metallothionein compared to zinc may allow it displace zinc in the tissues (Mertz, 1986).

The serum vitamin A levels were not affected by PA consumption (Table 3.17). The concentration of liver vitamin A remained normal with the exception of the group fed TR without added copper or vitamin A. In this group the liver vitamin A content was elevated (Table 3.17). Buckmaster et al.(1977) showed that Japanese quail have a very low rate of *in vitro* pyrrole production. In Japanese quail, the liver may have detoxification enzymes against PA toxicosis. The results obtained from the TR without supplement group may be due to inhibition of PA on the mobilization of vitamin A.

Copper supplementation of the diet induced a gross increase in serum vitamin A, whereas Rechman et al.(1987) reported that in copper deficient rats, retinol and retinyl esters increased significantly in the liver and decreased in the serum. They concluded that copper has an involvement in the synthesis of vitamin A transport proteins. This may also explain the above results.

Ceruloplasmin activity was not affected by PA exposure (Figure 3.25). The consistency of serum copper and vitamin A levels may be one of the reasons, in which both copper and vitamin A have been considered to regulate the ceruloplasmin synthesis (Barber and Cousins, 1987).



Table 3.12 Experimental design for Japanese quail

Treat. No.	Basal diet(%)	Ground corn(% diet)	Tansy ragwort(% diet)	Copper (ppm)	Vitamin A (IU/kg diet)	birds/ treat.
1	90	10	0	0	0	12
2	90	0	10	0	0	11
3	90	10	0	250	0	11
4	90	0	10	250	0	11
5	90	10	0	0	25,000	11
6	90	0	10	0	25,000	11
7	90	10	0	250	25,000	11
8	90	0	10	250	25,000	11

Table 3.13 Composition of the basal diet (OSU #1876)

ingredients	1876(%)
Yellow corn	47.00
Soybean meal	41.40
Fish meal-herring	2.50
Alfalfa meal	3.00
Meat and bone meal	5.00
Defluo. phosphate	0.50
Salt	0.25
Trace mineral (mix-65)*	0.05
Vitamin premix (I-75)*	0.20
D, L-methionine	0.10
Total	100.00

\* The contents of premixes are the same as above (Table 3.1.2).

Table 3.14 Growth and feed efficiency of Japanese quail fed copper, vitamin A and tansy ragwort

Deitary treatment			Ave. initial wt.(g)	Ave. final wt.(g)	Ave. weekly gain (g)	Feed efficiency (F/G)*
% dietary tansy ragwort	Cu (ppm)	Vitamin A (IU/kg)				
0	0	0	22.13±1.11	131.98±5.76	18.31±0.89 <sup>a</sup>	5.340
10	0	0	22.11±1.03	113.94±3.64	15.31±0.56 <sup>ab</sup>	6.750
0	250	0	22.08±0.90	121.02±5.23	16.49±0.80 <sup>ab</sup>	6.260
10	250	0	22.69±1.04	118.04±3.73	15.89±0.65 <sup>ab</sup>	6.500
0	0	25,000	22.20±0.98	126.29±4.85	17.35±0.83 <sup>ab</sup>	5.520
10	0	25,000	22.49±0.85	118.33±3.98	15.97±0.65 <sup>ab</sup>	6.683
0	250	25,000	22.43±1.11	129.98±5.28	17.93±0.83 <sup>ab</sup>	5.420
10	250	25,000	22.91±1.28	112.33±4.92	14.76±0.53 <sup>b</sup>	6.340

Means ± SE in the same followed by different superscripts are different (P < 0.05)

\* feed intake (g)/ body weight (g)

Table 3.15 Copper concentration and ceruloplasmin in serum and liver of Japanese quail fed copper, vitamin A and tansy ragwort

Dietary treatment			Serum copper (ppm)	Liver copper (ppm)(wet tissue)	Ceruloplasmin (U/ml)
% dietary tansy ragwort	Cu (ppm)	Vitamin A (IU/kg)			
0	0	0	0.340±0.04	6.49±0.74 <sup>a</sup>	24.6±3.3
10	0	0	0.297±0.03	4.62±0.44 <sup>ab</sup>	28.1±4.3
0	250	0	0.305±0.04	5.75±0.60 <sup>ab</sup>	22.3±5.4
10	250	0	0.318±0.01	5.75±0.91 <sup>ab</sup>	23.0±7.5
0	0	25,000	0.292±0.01	5.62±0.72 <sup>ab</sup>	24.4±2.1
10	0	25,000	0.298±0.03	3.71±0.34 <sup>b</sup>	24.0±3.9
0	250	25,000	0.335±0.02	5.30±0.48 <sup>ab</sup>	22.2±1.8
10	250	25,000	0.310±0.02	4.07±0.12 <sup>ab</sup>	26.7±6.7

Means ± SE in the same followed by different superscripts are different (P < 0.05)

Table 3.16 Zinc and iron concentration in serum and liver (wet tissue) of Japanese quail fed copper, vitamin A and tansy ragwort

Dietary treatment			Serum zinc (ppm)	Liver zinc (ppm)	Serum iron (ppm)	Liver iron (ppm)
% dietary tansy ragwort	Cu (ppm)	Vitamin A (IU/kg)				
0	0	0	2.80±0.45 <sup>ab</sup>	31.16±1.81 <sup>a</sup>	7.87±0.63	236.01±25.78
10	0	0	3.38±0.59 <sup>ab</sup>	27.84±1.66 <sup>ab</sup>	8.83±1.08	253.72±25.40
0	250	0	3.43±0.59 <sup>ab</sup>	21.18±1.80 <sup>b</sup>	9.08±0.98	169.81±32.67
10	250	0	2.72±0.40 <sup>ab</sup>	23.94±2.76 <sup>ab</sup>	7.35±0.48	181.22±31.11
0	0	25,000	2.23±0.17 <sup>b</sup>	26.40±3.53 <sup>ab</sup>	8.82±0.80	156.81±15.66
10	0	25,000	3.35±0.73 <sup>ab</sup>	25.29±1.78 <sup>ab</sup>	9.45±1.40	248.26±25.94
0	250	25,000	4.55±0.38 <sup>a</sup>	21.20±1.51 <sup>b</sup>	7.47±0.63	169.91±19.80
10	250	25,000	3.52±0.40 <sup>ab</sup>	21.45±0.63 <sup>b</sup>	8.17±0.59	237.27±26.94

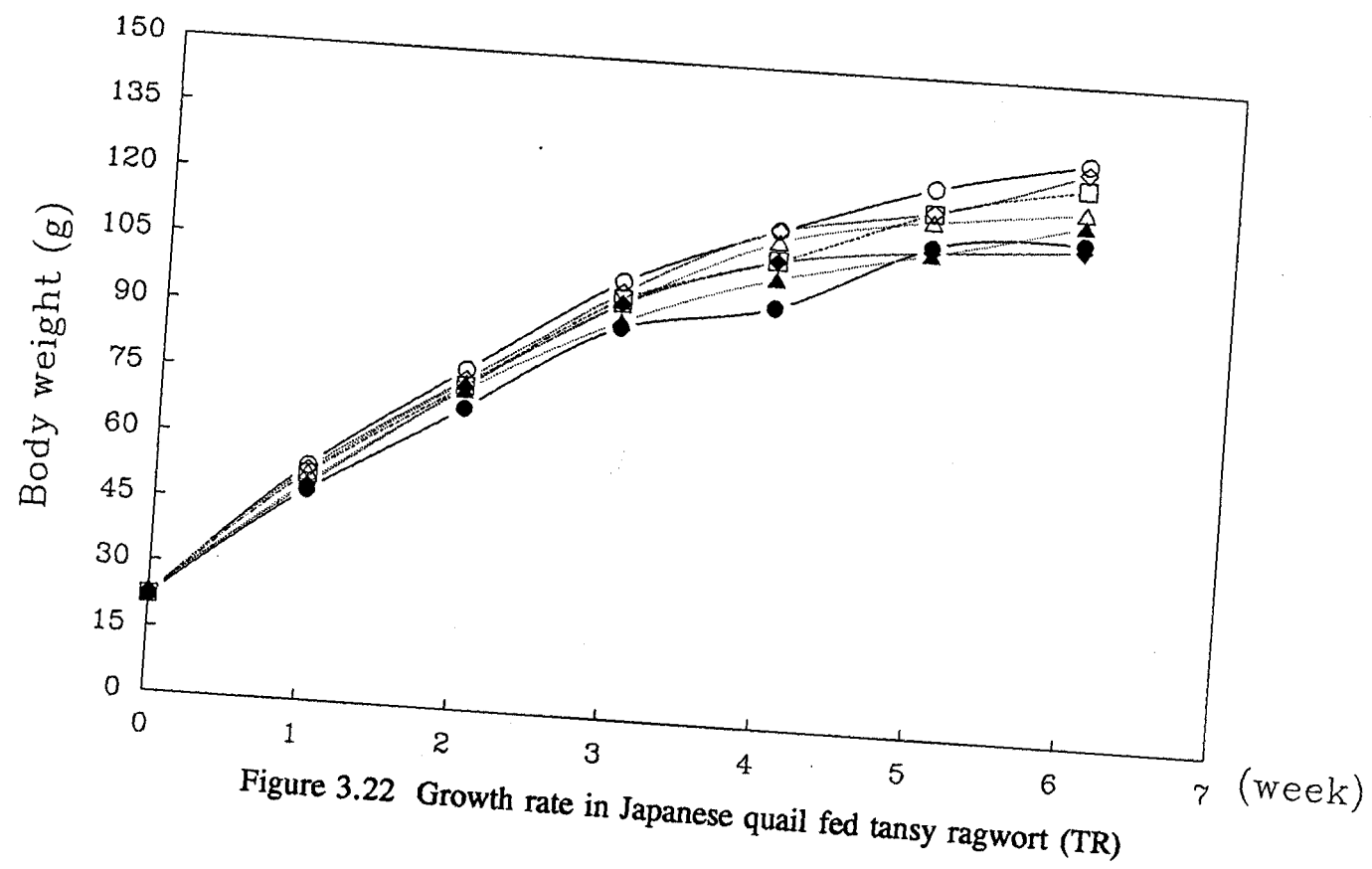
Means ± SE in the same followed by different superscripts are different (P < 0.05)

Table 3.17 Liver weights and vitamin A concentration in serum and livers of Japanese quail fed copper, vitamin A and tansy ragwort

Dietary treatment			Liver weight (g)	Serum vitamin A (IU/ml)	Liver vitamin A* (IU/g)(wet tissue)
%dietary tansy	Cu (ppm)	Vitamin A (IU/kg)			
0	0	0	3.22±0.40	4.306±0.424 <sup>c</sup>	160.62±36.32 <sup>a</sup>
10	0	0	2.83±0.17	3.948±0.638 <sup>c</sup>	524.40±80.85 <sup>b</sup>
0	250	0	3.55±0.48	5.121±0.253 <sup>bc</sup>	148.27±25.47 <sup>a</sup>
10	250	0	3.08±0.38	4.206±0.399 <sup>c</sup>	171.09±40.71 <sup>a</sup>
0	0	25,000	3.13±0.26	7.858±0.665 <sup>ab</sup>	3235.63±576.26 <sup>c</sup>
10	0	25,000	3.02±0.24	7.258±0.458 <sup>abc</sup>	3466.54±584.59 <sup>c</sup>
0	250	25,000	3.52±0.52	9.836±1.68 <sup>a</sup>	4266.84±733.99 <sup>c</sup>
10	250	25,000	3.45±0.31	9.797±1.08 <sup>a</sup>	4901.76±206.45 <sup>c</sup>

Means ± SE in the same followed by different superscripts are different (P < 0.05)

\* Comparison was made in Log values.



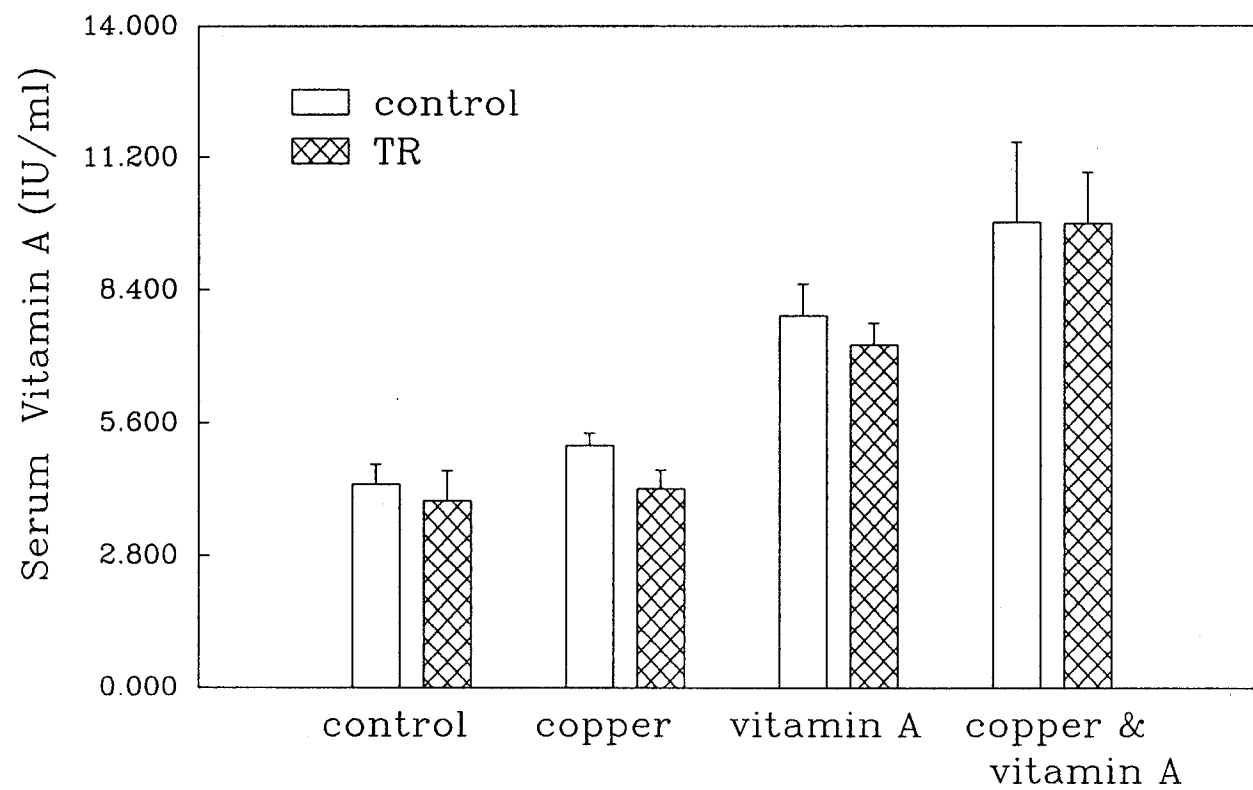


Figure 3.23 Serum vitamin A levels in Japanese quail fed tansy ragwort (TR)



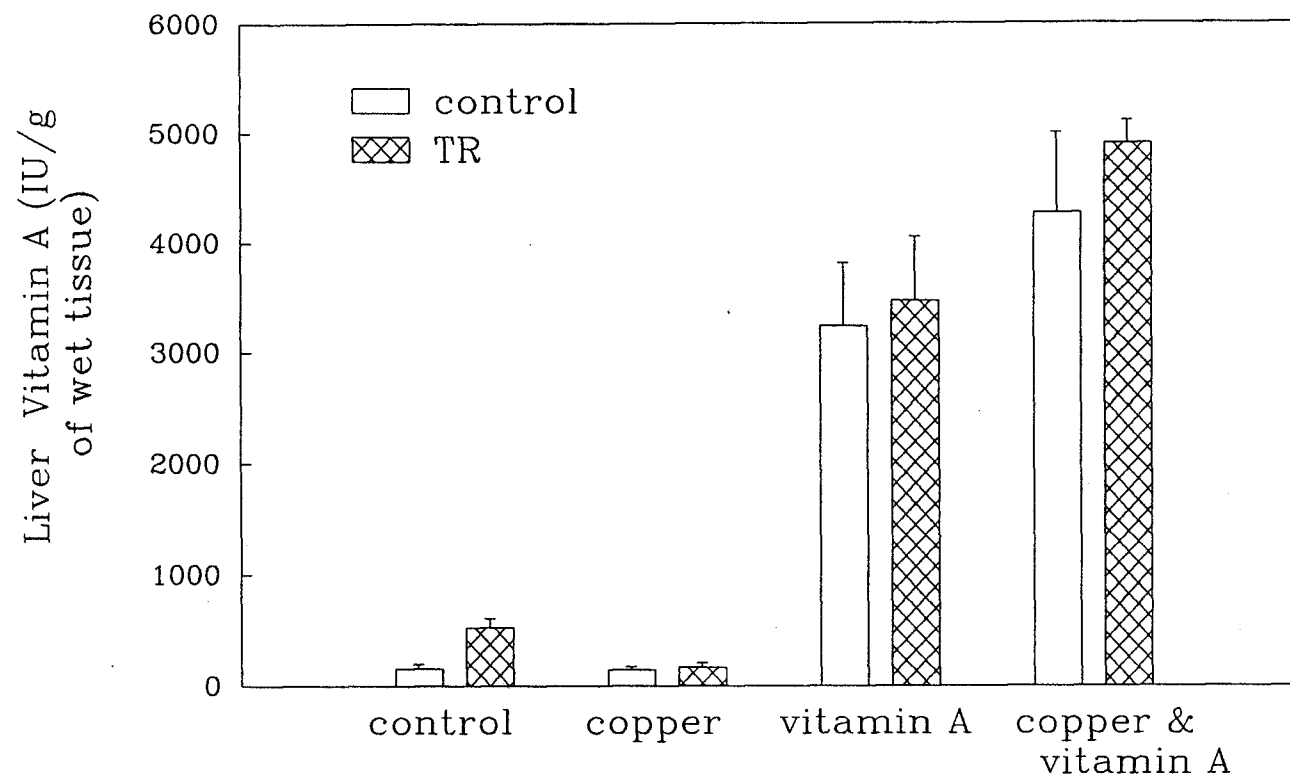


Figure 3.24 Liver vitamin A levels in Japanese quail fed tansy ragwort (TR)

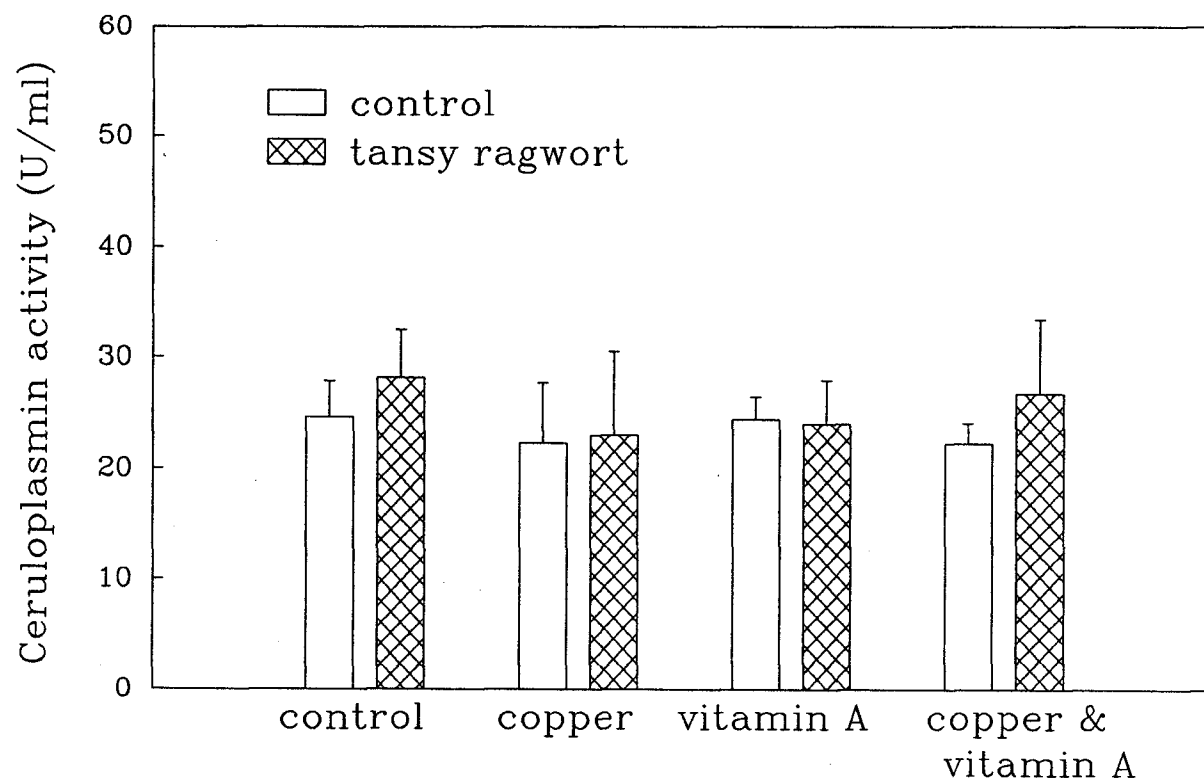


Figure 3.25 Serum ceruloplasmin activity in Japanese quail fed tansy ragwort

## Conclusions and Suggestions for Further Research

Pyrrolizidine alkaloids (PA) in tansy ragwort (*Senecio jacobaea*) have pronounced effects on blood and liver levels of copper and vitamin A in the chicken, but not in Japanese quail. The effects of PA on vitamin A metabolism occurred in the early stages of PA toxicosis. These results may be a reflection of inhibition of hepatic synthesis of proteins involved in vitamin A metabolism such as retinol-binding protein and impairment of mobilization of vitamin A in the liver. The reductions in serum vitamin A levels in PA toxicosis seem to be unique. The only other situation in which reduced blood vitamin A levels are noted is with dietary vitamin A deficiency. It is possible that some of the signs of PA toxicity, such as the effects on growth, may in part be due to a cellular vitamin A deficiency caused by the reduced blood level of the vitamin A. Further research is necessary to elucidate the mode of action of PA on nutrient metabolism. Examples of aspects which should be examined to elucidate these interactions include:

### 1) vitamin A and PA

- a).  $\beta$ -carotene conversion to retinol
- b). cellular distribution of vitamin A as influenced by hepatotoxic effects of PA
- c). synthesis of retinol-binding protein
- d). serum lipoproteins and vitamin A transport
- e). interaction with vitamin E

### 2) copper and PA

- a). liver copper mobilization

- b). PA and metallothionein levels
- c). other copper binding proteins
- d). mineral absorption

The possible involvement of lack of cellular vitamin A or antioxidants such as vitamin E on the development of PA-induced tissue damage should be assessed, to determine if the marked effects of PA on tissue levels of vitamin A are of biological significance or are merely a reflection of effects of PA on protein synthesis.

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## **APPENDICES**

PLATE A: chicken liver --- normal hepatic tissue

PLATE B, C, D: chicken liver --- showing effect of PA in hepatic tissue

PLATE E: chicken liver --- showing effect of PA in hepatic tissue of chicks  
pre-fed a high vitamin A diet followed by  
a TR-containing diet

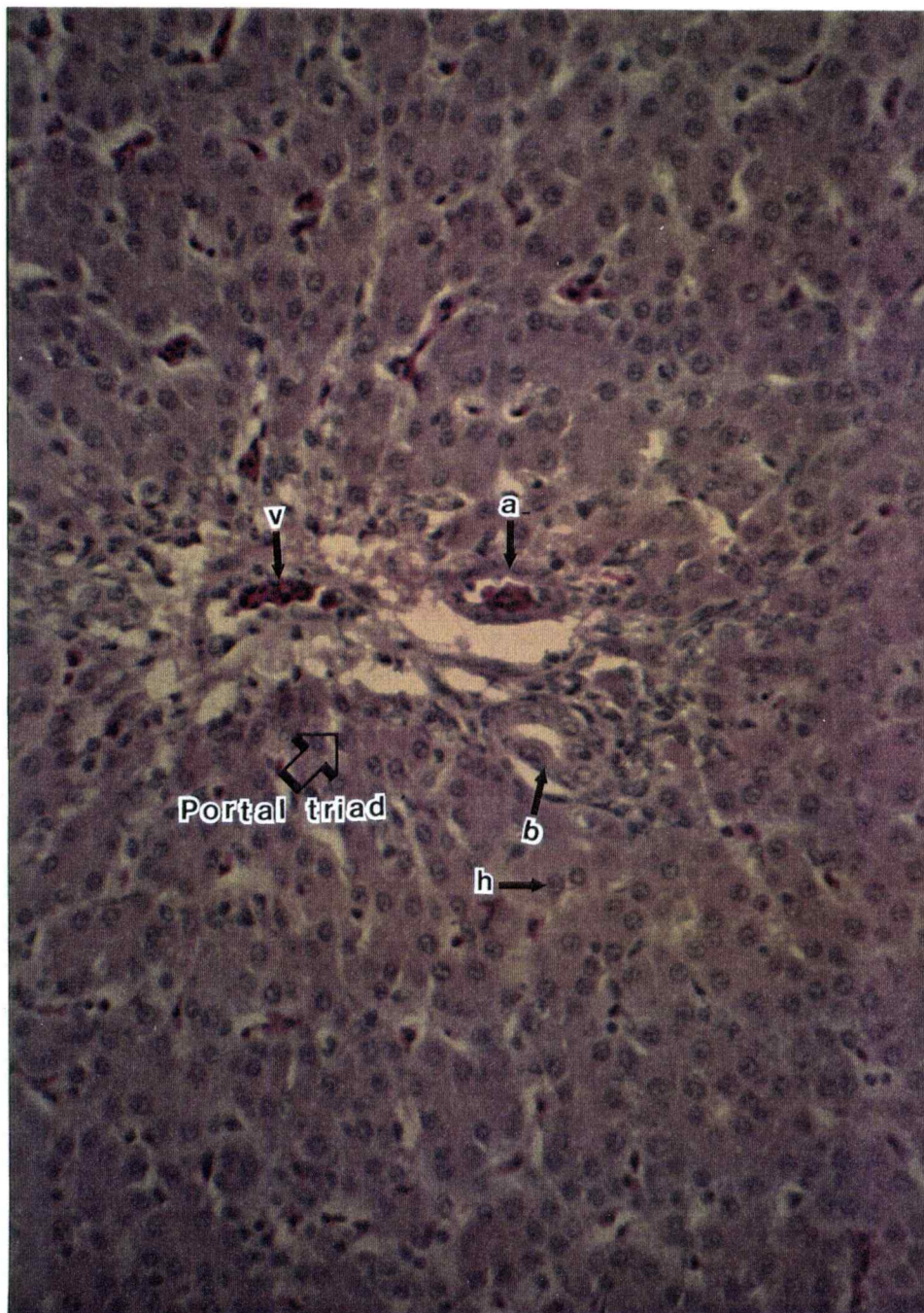


Plate A. Chicken liver --- showing the normal hepatic tissue  
with a portal triad

v = hepatic vein; a = hepatic artery;  
b = bile duct; h = hepatocyte;



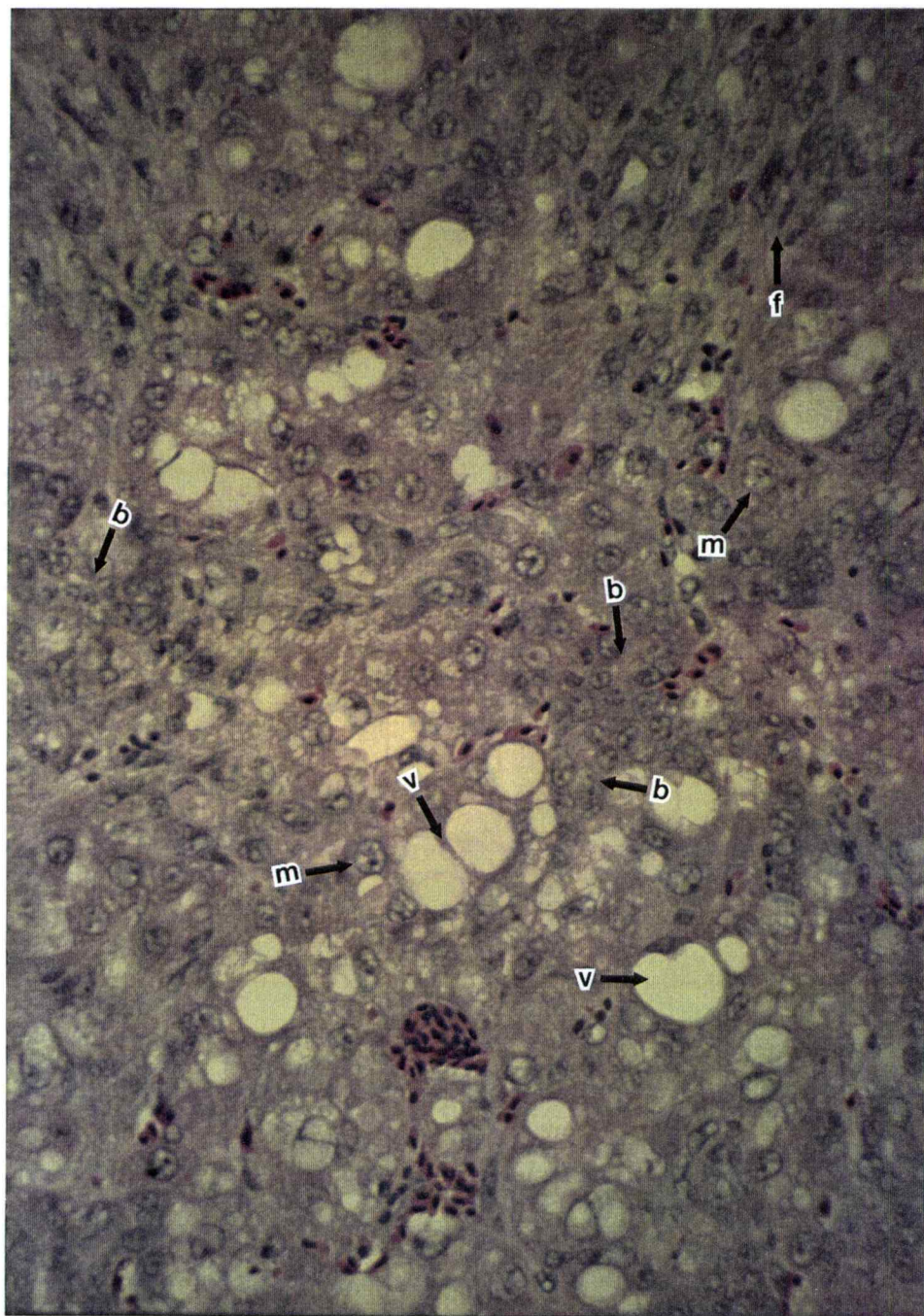


Plate B. Chicken liver --- showing effect of pyrrolizidine alkaloid  
in hepatic tissue

v = vacuolated hepatocyte; b = bile duct hyperplasia;  
m = megalocyte; f = fibrosis;



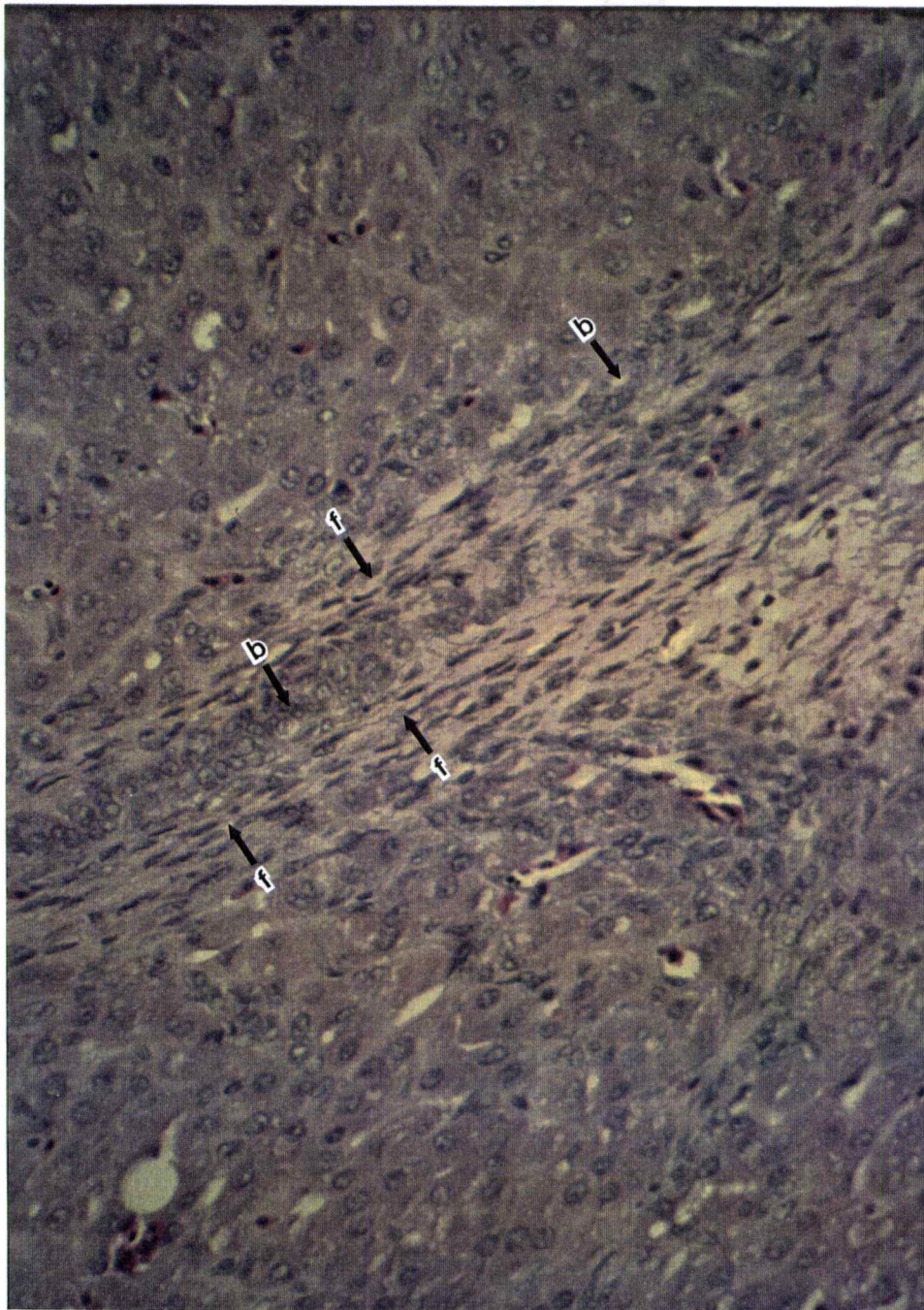


Plate C. Chicken liver --- showing effect of pyrrolizidine alkaloid  
in hepatic tissue

b = bile duct hyperplasia; f = fibrosis;



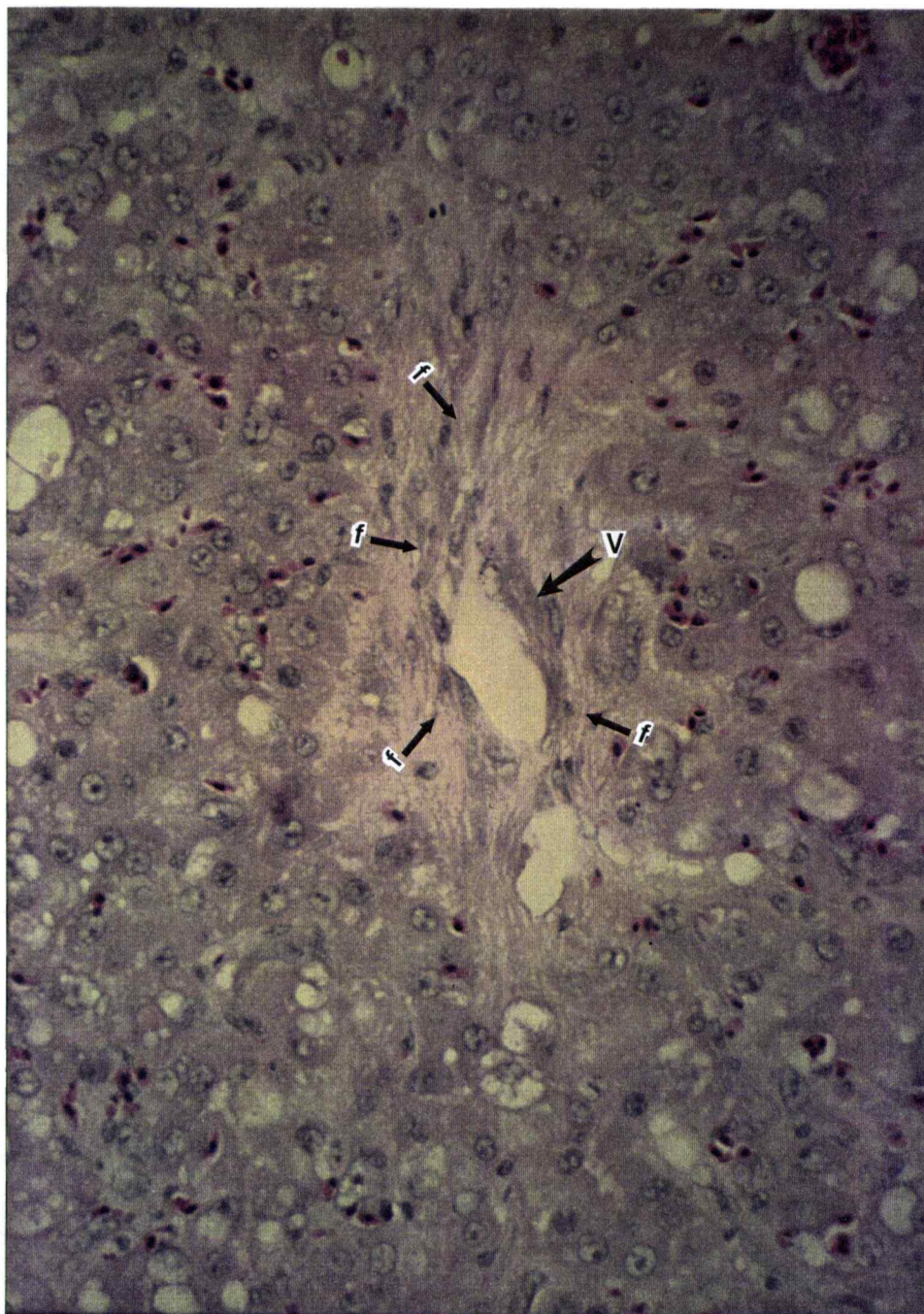


Plate D. Chicken liver --- showing effect of pyrrolizidine alkaloid  
in hepatic tissue with fibrosis around centrilobular vein  
resulting in veno-occlusion  
v = centrilobular vein; f = fibrosis;



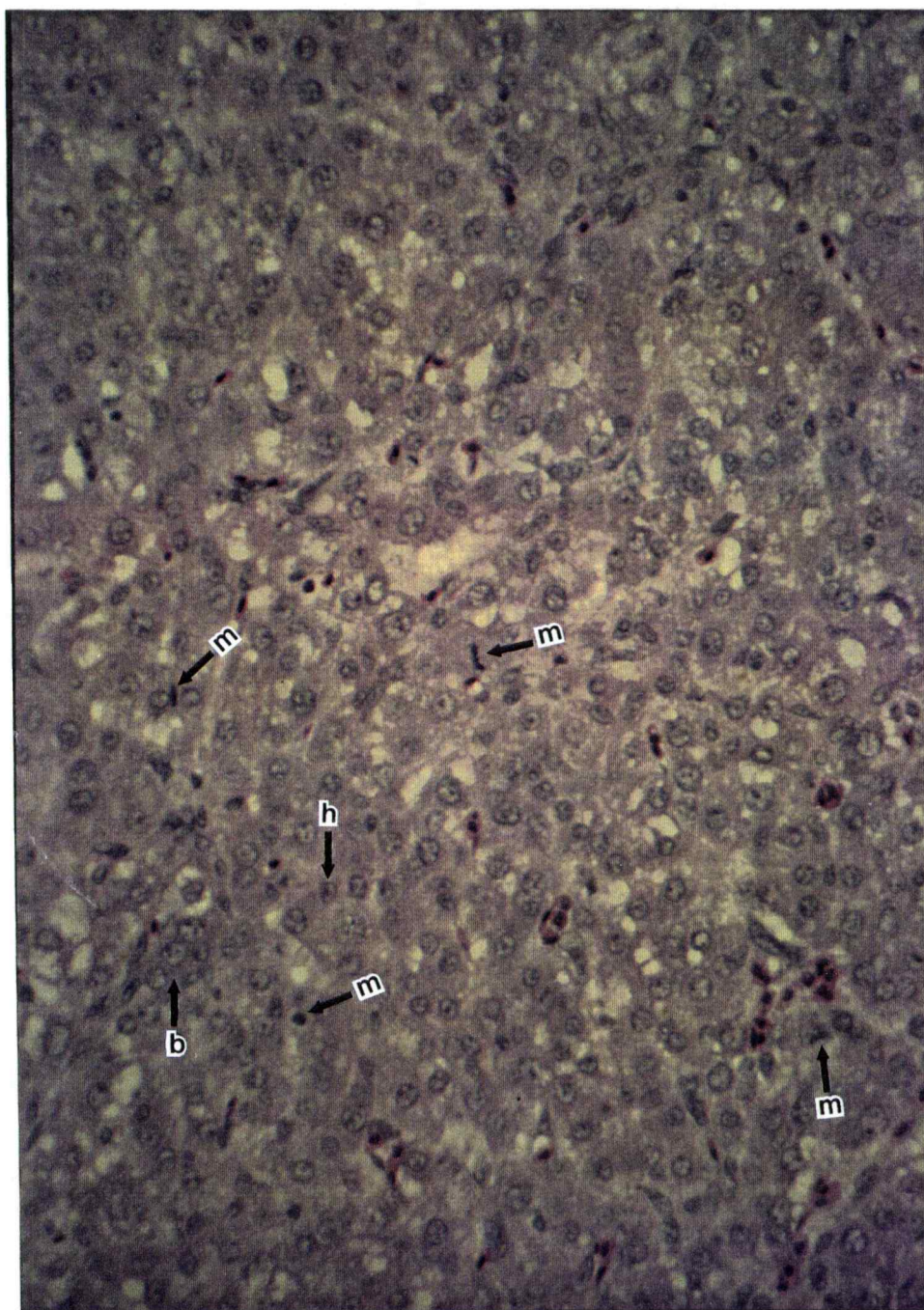


Plate E. Chicken liver --- showing effect of pyrrolizidine alkaloid  
in chicks pre-fed a high vitamin A diet followed  
by a tansy ragwort-containing diet  
h = hepatocyte; b = bile duct; m = mitotic figure;